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# **IDIOPATHIC POLYNEUROPATHY – A PATHOGENETIC STUDY**

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# IDIOPATHIC POLYNEUROPATHY – A PATHOGENETIC STUDY

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To Sebastian and Matilda with love



## ABSTRACT

Polyneuropathy (PNP) stands for dysfunction in the peripheral nerves. The classical clinical manifestations of PNP are distal, symmetric sensory symptoms, such as numbness and pain, as well as distal weakness in a sock- and glove pattern. For most patients with PNP, the progression rate is slow and the degree of impairment is limited. Despite that, health-related quality of life is negatively affected in patients with PNP regardless of etiology. Further, PNP has a negative impact on daily activities at home, leisure and work. Patients with PNP have an increased risk of falls and fall-related injuries due to disturbed balance and walking difficulties. Polyneuropathy is a common neurological disorder with a prevalence of 1.6%. The frequency increases with age and is about 6.5% among the population older than 60 years. The etiology of PNP varies from systemic disorders, infections, hereditary, inflammatory, toxic to vitamin deficiencies etc. The most common identifiable cause of PNP is diabetes mellitus. About 25% of the PNP remain idiopathic despite a thorough etiological investigation. This is particularly common for patients with small fiber neuropathy (SFN) and for patients with chronic idiopathic axonal polyneuropathy (CIAP). There is no pharmaceutical treatment to offer patients with idiopathic PNP, since there is no cure for diffusely dysfunctional peripheral axons. Therefore, there is a need to further explore possible pathogenesis of idiopathic PNP, to hopefully in the future being able to modulate the condition.

There are several hypotheses for the pathogenesis of idiopathic PNP, such as underdiagnosed hereditary disorders, microangiopathy in the microvessels in the vasa nervorum, axon-degenerative factors and disturbed mitochondrial dynamics in the peripheral nerves. This thesis is based on four studies with the overall aim to explore possible etiologies of idiopathic PNP by probing into idiopathic PNP from different angles.

In Study I we aimed to explore the etiological causes of seemingly idiopathic SFN by applying a standardized focused investigation and to investigate if late-onset Fabry disease could be a cause of idiopathic SFN. Forty-five adults younger than 60 years with seemingly idiopathic pure or predominantly SFN underwent a standardized focused investigation and a potential etiology was identified in 12 patients, with the most frequent being impaired glucose tolerance (58.3%, 7 of 12 patients). The patients deemed to have a true idiopathic SFN were offered genetic screening of the  $\alpha$ -Galactosidase A *GLA* gene (Fabry disease). Four patients declined. Genetic alterations of unknown clinical significance in *GLA* were detected in 6 of the 29 patients with idiopathic SFN, but this rate did not differ significantly from that in healthy controls (n=203). All patients with genetic alterations of unknown clinical significance had in the end normal biomarkers and clinical evaluation in selected cases showed no Fabry-specific manifestations in other organs. Idiopathic SFN in young to middle-aged Swedish patients does not seem to be due to late-onset Fabry disease.

Study II was a retrospective histopathological ultrastructural study in which we examined the

presence of microangiopathy and autophagy-related structures in sural nerve biopsies of ten patients with CIAP, eleven controls with inflammatory neuropathy and ten normal controls without sensory polyneuropathy. No significant difference in endoneurial microangiopathic parameters in patients with CIAP compared to normal controls were identified. Hence, our results do not support the hypothesis that CIAP is primarily caused by a microangiopathic process in endoneurial microvessels in peripheral nerves. However, a significantly higher density of endoneurial autophagy-related structures, particularly in patients with CIAP but also in patients with inflammatory neuropathy, compared to normal controls was detected. The present study confirmed a previous study that the autophagic machinery is activated in human peripheral nerve. However, whether the alteration in the autophagy pathway is a consequence or a cause of the neuropathy is not clear.

Study III was a register-based nation-wide nested case-control study in which we included 2659 patients with CIAP identified from the Swedish Patient Register during the period 2001-2010 and 13295 age- and sex-matched controls to assess the associations of mitochondrial disease (MD), vascular dementia (VD) and Alzheimer's disease (AD) with the subsequent risk of CIAP. To reassure that our CIAP cases were truly idiopathic, all individuals with comorbid diseases known to be associated with PNP were excluded from the study base. Individuals with MD had a four-fold increased risk of subsequent CIAP, whereas individuals with VD and AD had a decreased risk (OR 0.17, 95% CI 0.04-0.69; OR 0.18, 95% CI 0.06-0.59). The lower risks of VD and AD before CIAP might be due surveillance bias, i.e. reduced investigation of disturbed balance and walking difficulties among patients with dementia. We also conducted a follow-up study of the cases and controls to assess the risk of MD, VD or AD among patients with CIAP, in comparison to individuals without CIAP. Patients with CIAP had a nine-fold increased risk of subsequent MD and a two-fold increased risk of VD, but no increased risk of AD, compared to individuals without CIAP. This results might indicate that microangiopathy in the PNS and CNS do co-exist.

Study IV was a multicenter study in which we aimed to explore the value of genetic screening for Fabry disease and hereditary ATTR amyloidosis in patients with idiopathic SFN or mixed neuropathy in a clinical setting in the Nordic region. In total 172 patients were enrolled in the study at nine participating neurological departments from four different Nordic countries. Seventeen patients were excluded due to exclusion factors found out later in the screening process. Genetic sequencing of the *TTR* gene and *GLA* gene was performed in 155 patients. No pathogenic mutations in the *TTR* gene were found. A single patient did have an earlier described possible pathogenic variant, R118C, in the *GLA* gene, but the clinical investigation showed no firm signs of Fabry disease. In conclusion, screening for hereditary ATTR amyloidosis and Fabry disease in patients with idiopathic small SFN or mixed neuropathy without any additional disease-specific symptoms or clinical characteristics in a Nordic population seems not to be of value in a clinical setting.



## LIST OF SCIENTIFIC PAPERS

- I. Idiopathic small fiber neuropathy: phenotype, etiologies and the search for Fabry disease. **Kristin Samuelsson**, Konstantinos Kostulas, Magnus Vrethem, Arndt Rolfs, Rayomand Press.  
*Published in J Clin Neurol. 2014 April;10(2):108-118*
- II. Study of autophagy and microangiopathy in sural nerves of patients with chronic idiopathic axonal polyneuropathy. **Kristin Samuelsson**, Ayman A.M. Osman, Maria Angeria, Mårten Risling, Simin Mohseni, Rayomand Press.  
*Published in PLoS One. 2016 Sep 23;11(9):e0163427*
- III. Comorbidity of mitochondrial disease and dementia in patients with idiopathic polyneuropathy. **Kristin Samuelsson**, Daniela Mariosa, Fang Fang, Rayomand Press.  
*Accepted manuscript. Eur J Neurol. 2018 Mar 5. doi: 10.1111/ene.13612. [Epub ahead of print]*
- IV. No firm cases of Fabry disease and hereditary ATTR amyloidosis identified in a multicenter genetic screening study of patients with idiopathic polyneuropathy in the Nordic region. **Kristin Samuelsson**, Ana Radovic, Rayomand Press, Mari Auranen, Emil Ylikallio, Henna Tynismaa, Mikko Kärppä, Matilda Veteläinen, Niina Peltola, Svein Ivar Mellgren, Åse Mygland, Chantal Tallaksen, Henning Andersen, Astrid Juhl Terkelsen, Freja Fontain, Aki Hietaharju.  
*Manuscript*

# CONTENTS

1	Background.....	1
1.1	Epidemiology .....	1
1.2	Subcategories of polyneuropathy.....	1
1.2.1	Definition of small fiber neuropathy .....	2
1.3	Symptoms and clinical findings in patients with polyneuropathy.....	3
1.3.1	Symptoms and clinical findings in small fiber neuropathy .....	3
1.4	How to diagnose polyneuropathy .....	4
1.4.1	Assessment of large fiber affection .....	5
1.4.2	Assessment of small fiber affection.....	5
1.5	Etiology of polyneuropathy.....	7
1.5.1	Etiology of axonal polyneuropathy .....	7
1.5.2	Etiology of small fiber neuropathy .....	9
1.5.3	Fabry disease as a cause of small fiber neuropathy .....	9
1.6	Idiopathic polyneuropathy .....	11
1.6.1	Idiopathic small fiber neuropathy.....	12
1.6.2	Chronic idiopathic axonal polyneuropathy .....	13
1.7	Possible hypotheses for the genesis of idiopathic polyneuropathy.....	14
1.7.1	Microangiopathy - a pathogenic cause of chronic idiopathic axonal polyneuropathy? .....	14
1.7.2	Neurodegeneration as an alternative hypothesis for the pathogenesis of chronic idiopathic axonal polyneuropathy.....	17
1.7.3	Mitochondrial dysfunction - a possible role in the pathogenesis of polyneuropathy.....	19
2	Aims.....	21
2.1	Specific aims.....	21
3	Material.....	23
3.1	Patient population Study I and IV.....	23
3.1.1	Retrospective part.....	23
3.1.2	Prospective part.....	24
3.1.3	Inclusion process.....	24
3.2	Patient population Study II.....	24
3.3	Patient population study III.....	25
3.3.1	Study base.....	25
3.3.2	Identifying CIAP patients and definitions of MD, VD and AD.....	25
3.4	Controls.....	26
3.4.1	Controls for the genetic analysis in Study I .....	26
3.4.2	Controls in the sural nerve biopsy study (Study II) .....	26
3.4.3	Controls in the register-based study (Study III) .....	26
3.5	Ethical approval and consent .....	26
4	Methods .....	27
4.1	The assessment of small and large fiber neuropathy.....	27

4.1.1	Study I and IV .....	27
4.1.2	Study II .....	27
4.2	Definition of the idiopathic nature of the polyneuropathy .....	27
4.2.1	Standardized focused investigation in Study I .....	27
4.2.2	Nerve biopsy study (Study II).....	27
4.2.3	Nested case-control and cohort study (Study III).....	27
4.2.4	Nordic screening study of FD and hATTR amyloidosis in patients with idiopathic SFN (Study IV).....	29
4.3	Genetic analysis .....	29
4.4	Biochemical analyses for Fabry disease .....	29
4.5	Sural nerve biopsy analysis .....	29
4.5.1	Parameters assessed in semi-thin sections (light microscopy) .....	30
4.5.2	Parameters assessed in ultra-thin sections (electron microscopy) .....	30
4.6	Study III .....	31
4.6.1	Nested case-control study .....	31
4.6.2	Matched cohort study .....	32
4.6.3	Sensitivity analysis .....	32
4.6.4	Validation of definitions of vascular dementia and Alzheimer's disease.....	32
4.7	Statistical analysis.....	33
4.7.1	Study I, II and IV .....	33
4.7.2	Study III.....	33
5	Results.....	34
5.1	The genetic screening studies (Study I and IV).....	34
5.1.1	Demographic and clinical characteristics .....	35
5.1.2	Results of the standardized focused investigation in Study I .....	36
5.1.3	Genetic screening for Fabry disease in patients with idiopathic small fiber neuropathy .....	37
5.1.4	Genetic screening of hATTR amyloidosis in patients with idiopathic small fiber neuropathy .....	38
5.2	Sural nerve biopsy study (Study II) .....	38
5.2.1	Demographics and clinical data.....	38
5.2.2	Results of analysis of sural nerve biopsies .....	40
5.3	Register-based study (Study III) .....	44
5.3.1	Mitochondrial disease, vascular dementia and Alzheimer's disease and the subsequent risk of CIAP (nested case-control study).....	44
5.3.2	CIAP and the subsequent risk of mitochondrial disease, vascular dementia and Alzheimer's disease (matched cohort study).....	44
5.3.3	Sensitivity analysis .....	44
6	Discussion.....	46
6.1	Morbidity of idiopathic polyneuropathy.....	46
6.2	Reevaluating patients with idiopathic polyneuropathy .....	46

6.2.1	Is impaired glucose tolerance a cause of neuropathy?.....	47
6.3	Is there a value of genetic screening of rare diseases in patients with idiopathic polyneuropathy?.....	48
6.3.1	Fabry disease .....	48
6.3.2	Hereditary ATTR amyloidosis .....	50
6.4	Is microangiopathy involved in the pathogenesis of idiopathic polyneuropathy .....	50
6.4.1	The unexpected findings in vasculitic neuropathy .....	52
6.4.2	The possible association between microangiopathy in the PNS and CNS .....	52
6.5	The potential role of autophagy in polyneuropathy .....	53
6.6	Is there an association between mitochondrial disease and idiopathic polyneuropathy? .....	54
6.7	Limitations .....	54
6.7.1	Limitations in Study I and IV .....	54
6.7.2	Limitations in Study II .....	55
6.7.3	Limitations in Study III.....	56
7	Conclusions .....	58
8	Future perspectives.....	59
9	Populärvetenskaplig sammanfattning.....	61
10	Acknowledgements .....	64
11	References .....	67

## LIST OF ABBREVIATIONS

AD	Alzheimer's Disease
AIDP	Acute Inflammatory Demyelinating Polyneuropathy
ALS	Amyotrophic Lateral Sclerosis
ARS	Autophagy-Related Structures
BA	Basal membrane area
BLAT	Basal Lamina Area Thickness
CIAP	Chronic Idiopathic Axonal Polyneuropathy
CIDP	Chronic Inflammatory Demyelinating Polyneuropathy
CMT	Charcot-Marie-Tooth
CNS	Central Nervous System
COPD	Chronic Obstructive Pulmonary Disease
Drp1	Dynamin-Related Protein 1
EA	Endothelial cell Area
EFNS	European Federation of Neurological Societies
FD	Fabry Disease
$\alpha$ -GAL	$\alpha$ -Galactosidase A
Gb <sub>3</sub>	Globotriaosylceramide
GLA	$\alpha$ -Galactosidase A gene
hATTR	Hereditary ATTR
HMSN	Hereditary Motor Sensory Neuropathy
HSAN	Hereditary Sensory and Autonomic Neuropathy
ICD	International Classification of Diseases
IENFD	Intra-Epidermal Nerve Fiber Density
IGT	Impaired Glucose Tolerance
LA	Lumen Area
LD	Length-Dependent
LEA	Lumen and Endothelial cell Area
Lyso-Gb <sub>3</sub>	Globotriaosylsphingosine
MD	Mitochondrial Disease
MFN2	Mitofusin-2

MGUS	Monoclonal Gammopathy of Underdetermined Significance
MLPA	Multiplex Ligation-Probe Assay
MS	Multiple Sclerosis
NLD	Non-Length-Dependent
OGTT	Oral Glucose Tolerance Test
PNP	Polyneuropathy
PNS	Peripheral Nervous System
QST	Quantitative Sensory Testing
SFN	Small Fiber Neuropathy
TIA	Transitory Ischemic Attack
TTR	Transthyretin
VA	Vessel Area
VD	Vascular Dementia
WMC	White Matter Changes

# 1 BACKGROUND

Polyneuropathy (PNP) stands for dysfunction in the peripheral nerves. The classical clinical manifestations of PNP are distal, symmetric sensory symptoms and distal weakness in a sock- and glove pattern. For most of the patients with PNP, the progression rate is slow and the degree of impairment is limited. Despite that, health-related quality of life is negatively affected in patients with PNP regardless of etiology (1-6). A Swedish questionnaire study has shown that PNP has a negative impact on daily activities at work, leisure time and household (7). In addition, chronic PNP is associated with walking difficulties and an increased risk of falls and fall-related injuries (8).

An epidemiological study from Denmark has shown that the diagnosis of PNP leads to negative socioeconomic consequences and an increased mortality rate (9). Patients with PNP had significantly higher health-related and social-transfer costs compared with a matched control group of people without a diagnosis of PNP. This burden was significantly raised even before diagnosis of PNP, presumably associated to comorbidities that causes PNP. Patients with PNP had lower employment rates, and interestingly the employed patients had lower incomes than employed controls (9).

## 1.1 EPIDEMIOLOGY

There are no epidemiological studies regarding the prevalence or the incidence of PNP in Sweden. Mygland et al. studied the prevalence of polyneuropathies in Vest-Agder in Norway and found a prevalence of 123 per 100 000 population (10). However, this study was hospital-based, reflecting the patients who were referred to a specialist. Consequently, the prevalence data probably are underestimated. A study from the US using the International Classification of Diseases (ICD)-codes found an overall prevalence of PNP of 1.66% (11). An incidence study from the Netherlands found an age-adjusted incidence rate of 77/100 000 person-years in persons aged 18 years and older (12). The overall incidence of PNP increases with age (12, 13). The prevalence in the population older than 55-60 years varies between 3.5-6.6% in different studies (11, 13, 14).

## 1.2 SUBCATEGORIES OF POLYNEUROPATHY

Polyneuropathy can be subdivided into different categories. This is of importance, not only from a research stand point, but also in the clinical routine work since it helps the clinician to select the etiological investigation that is relevant for each patient.

Polyneuropathy can be subdivided into large- and small fiber neuropathies (SFN) depending on fiber size of the affected nerves. Many patients have so called “mixed neuropathy”, where both large and small fibers are involved. In some patient categories, such as diabetic neuropathy, the PNP often start as a SFN, with an evolution of a large fiber neuropathy later on (15).

Large fiber neuropathy can be axonal or demyelinating, according to the primary site of injury. Mixed axonal and demyelinating polyneuropathies are the most common form of PNP encountered, since secondary damage on the axon or myelin is quite common irrespectively of the primary site targeted.

Depending on the type of nerve fibers affected, the patients develop different clinical symptoms (Table 1).

**Table 1. Subdivision of polyneuropathy depending on fiber size.**

	Large fiber neuropathy		Small fiber neuropathy	
<b>Fiber type sorted by thickness</b>	A $\alpha$ Myelinated	A $\beta$ Myelinated	A $\delta$ Myelinated	C Unmyelinated
<b>Function of fiber</b>	Motor Proprioception	Vibration Touch	Pain Cold	Pain Heat Autonomic nervous system
<b>Possible symptoms upon dysfunction</b>	Weakness Balance disorder	Reduced sensibility Paresthesia Numbness	Pain Insensitivity to cold	Pain Insensitivity to heat Autonomic symptoms

### 1.2.1 Definition of small fiber neuropathy

Many patients with SFN also have large fiber involvement in parallel. There is no consensus of the level of large fiber dysfunction that is permitted to co-exist in the diagnosis of SFN. However, the following definition has been recommended:

“A sensory neuropathy with paresthesia that are typically painful, along with abnormal findings of small fiber function in at least one of the following: neurological examination, specialized electrodiagnostic testing and pathologic studies” (16, 17).

Loss of vibration sensation at the toes and absent ankle reflexes may be included, but more significant signs of large fiber dysfunction, such as reduced proprioception at the toes,



generalized areflexia, loss of vibration at the ankle or above, distal weakness or abnormal nerve conduction studies, are not acceptable findings in patients with pure SFN (18).

Bakkers et al. stated a more recent definition:

“The diagnosis of SFN relies on clinical features (neuropathic pain and autonomic symptoms not otherwise explained; loss of pinprick and temperature sensation without signs of large-fiber dysfunction) combined with abnormal quantification of intra-epidermal nerve fiber density (IENFD) and/or deficit in temperature threshold testing” (1).

#### *1.2.1.1 Epidemiology of small fiber neuropathy*

In a study from the southern part of the Netherlands, Peters et al. presented a minimum incidence for isolated SFN of 11.7 cases/100 000/years and a minimum prevalence of 53 cases/100 000 (19). The incidence of SFN increases with age (19). Reports on the gender distribution in SFN have been conflicting, though most studies have found a female predominance (18-25).

### **1.3 SYMPTOMS AND CLINICAL FINDINGS IN PATIENTS WITH POLYNEUROPATHY**

The classic symptom is a distal, symmetric length-dependent (LD) sensory disturbance with numbness, tingling and sometimes pain in the feet, lower legs and hands with a slow rate of progress. Weakness does occur, but for most of the patients it is limited, especially in idiopathic polyneuropathies. In acute (AIDP) or chronic (CIDP) inflammatory demyelinating polyneuropathies, severe muscle weakness is common and affects proximal muscles with a high risk for aggravated disability. Similarly, patients with hereditary motor sensory neuropathies (HMSN), also called Charcot-Marie-Tooth (CMT), may have pronounced muscle weakness. Other rapidly progressive polyneuropathies are hereditary ATTR (hATTR) amyloidosis (previously named familial amyloid polyneuropathy (FAP) (26)) or PNP due to vasculitis. The latter should be considered if the patient has asymmetric symptoms or if the electrophysiology indicates a pattern of mononeuritis multiplex (27).

Gait disturbance is common and can be due to both muscle weakness and sensory dysfunction. If proprioception is affected the gait becomes ataxic. This is particularly common in neuronopathy or ganglionopathy, due to destruction of the dorsal root ganglia, which often is associated to Sjögren's syndrome or malignancy (28, 29). Other causes of ganglionopathy are toxic or infectious, but as many as 50% are idiopathic (30).

In the clinical examination of a patient with PNP, the typical findings are reduced or absent reflexes, distally reduced sensitivity for different modalities depending on affected fiber type (Table 1), distal weakness mainly in the foot and ankle and sometimes sensory ataxia.

#### **1.3.1 Symptoms and clinical findings in small fiber neuropathy**

Patients with SFN often have positive sensory symptoms described as tingling, burning, itching, prickling and pain, though pain is neither mandatory nor exclusive in SFN (16, 31).

Hypersensitivity is common and patients can have intolerance for clothes or sheets (31). Negative symptoms such as numbness, reduced pain and temperature sensibility do occur. In extreme cases, as for example in hereditary sensory and autonomic neuropathy (HSAN) V, a total insensitivity to pain and thermal sensations, leads to fractures and joint destructions (32).

The most common autonomic symptom is skin changes such as discoloration, dryness due to dysfunction in the sebaceous glands, altered skin temperature, excessive or lack of sweating (16, 33). Other symptoms are sicca symptoms i.e. dry mouth and eyes, erectile dysfunction, bladder or gastrointestinal dysfunction and orthostatic hypotension.

The symptoms in SFN are often LD, but can be patchy and diffuse i.e. non-length-dependent (NLD) SFN (34), or present as a focal neuropathy (mononeuropathy) or as a multifocal neuropathy (31). Patients with LD-SFN typically have leg-onset symmetrical sensory symptoms with a distal-to-proximal gradient, and a delayed spread of symptoms to the upper limbs, not seldom starting when the symptoms in the legs reach the knees (31). On the other hand, patients with NLD-SFN experience sensory symptoms in a rather patchy asymmetrical distribution in the face, trunk, and upper limbs, exclusively or in addition to symptoms in the lower limbs. The asymmetric pattern indicates that NLD-SFN represents a neuronopathy (35).

Interestingly, a small longitudinal study of patients with predominantly SFN (idiopathic or associated with impaired glucose tolerance (IGT) or diabetes), showed a decline in IENFD during follow-up time (2-3 years) irrespectively of location of biopsy (distal leg, distal thigh, proximal thigh) suggesting a NLD axonal degeneration pattern, instead of classical dying back LD phenomena (36).

Abnormal clinical signs in a patient with pure SFN may be scarce or absent (24). Reduced sensitivity to pain and temperature or hypersensitivity but intact strength, proprioception and reflexes are to be expected (16).

## **1.4 HOW TO DIAGNOSE POLYNEUROPATHY**

In addition to a typical patient history and clinical signs, the diagnosis of PNP relies on results of the electrophysiological and pathological investigations. Different questionnaires and clinical scales can be used both for research purpose and in the clinical work.

Unfortunately, no common scale is used by clinicians as in other neurological areas, such as Expanded Disability Status Scale (EDDS) in multiple sclerosis (MS), the National Institutes of Health Stroke Scale (NIHSS) in stroke or the Unified Parkinson's Disease Rating Scale (UPDRS) in Parkinson's disease. Instead there are a number of different scales with various alignments. In a recent review Hanewinkel et al. identified 27 scales (13 based on symptoms, eight on signs only and six on both symptoms and signs), all developed for a predefined PNP category (37).

## **1.4.1 Assessment of large fiber affection**

### *1.4.1.1 Nerve conduction studies (electroneurography)*

Electroneurography reveals large fiber function. The amplitude of axon potentials reflects the function of the axon, and typical findings in an axonopathy are low-amplitude sensory nerve action potentials and compound muscle action potentials, with no or slightly affected conduction velocities (38). In a demyelinating PNP the conduction velocities are slow and there can be prolonged F-wave latencies and distal latencies as well as conduction blocks. One method to assess peripheral nerve function in patients with PNP is electroneurography index, which is based on 12 electrophysiological parameters (conduction velocity, F-latency and amplitude) from five sensory and motor nerves from upper and lower extremities (39). The index can differentiate between axonal or demyelinating PNP. The European Federation of Neurological Societies (EFNS), nowadays European Academy of Neurology (EAN), has developed guidelines for electrophysiological criteria of the diagnosis of CIDP (40). For chronic idiopathic axonal polyneuropathy (CIAP) the pattern of the electroneurography should be axonal, but there are no corresponding guidelines for CIAP as for CIDP (41).

### *1.4.1.2 Sural nerve biopsy*

There are evidence-based guidelines on the processing and evaluation of peripheral nerve biopsies (42). However, there are no guidelines regarding indication and diagnostic value of a sural nerve biopsy in a clinical setting. The diagnostic yield is mainly in non-systemic vasculitis, i.e. a setting of an asymmetric painful sensorimotor axonal PNP or mononeuritis multiplex, or as a last way out to demonstrate amyloid if more accessible tissues have been found to be negative for amyloid (43, 44). However, sural nerve biopsy is an invasive procedure with a risk for permanent sensory deficiency and chronic pain. Other less invasive imaging tools, such as ultra-sound and MRI have started to become useful in clinical practice in inflammatory neuropathies (45), and might even be supportive in the diagnosis of vasculitic neuropathies and hATTR amyloidosis (46, 47).

## **1.4.2 Assessment of small fiber affection**

The diagnosis of SFN is debated as mentioned above. There is no golden standard for diagnosing SFN (31). The two most common methods to assess SFN are quantitative sensory testing (QST) and skin biopsy measuring IENFD. Quantitative sensory testing has been found to strongly correlate with IENFD according to the EFNS guidelines (48, 49). However, numerous studies favor skin biopsy before QST as a more precise method in diagnosing SFN (21, 24, 50). Nebuchennykh et al. suggest the two methods to be complementary, since QST assess the function of the small fibers, whereas skin biopsy is primarily a morphological evaluation (51).

Unfortunately, there are no Swedish laboratories that routinely perform analysis of skin biopsies, which explains why this method is not used in Sweden in clinical practice. For

research purpose a modified method of analyzing IENFD has been developed by associate professor Englund in Lund (52).

#### *1.4.2.1 Quantitative Sensory Testing*

Quantitative sensory testing measures the detection thresholds for warm and cold sensations. The method has some shortcomings since it requires cooperation from the patient. Also, abnormal results may indicate a disorder in the central nervous system (CNS) and not per definition in the peripheral nervous system (PNS) as illustrated in our case report (53). Furthermore, there has been a wide variation in the types of devices, settings, different testing algorithms in the different laboratories using QST (54). Bakkers et al. suggest recommendations to standardize the use of temperature threshold testing (54).

#### *1.4.2.2 Skin biopsy*

According to EFNS guidelines for diagnostic purposes in LD-SFN a 3 mm punch skin biopsy 10 cm above the lateral malleolus is recommended (55). In order to differentiate between LD-SFN and NLD-SFN an additional proximal biopsy from the thigh may be needed (55). Intra-epidermal nerve fibers crossing the dermal-epidermal junction should be counted and the analysis should be performed in a well-established skin biopsy laboratory. Bright-field immunohistochemistry or immunofluorescence with anti-PGP 9.5 antibodies is recommended for visualizing intra-epidermal nerve fibers (55).

However, IENFD only reflects the loss of fibers, not their possible dysfunction. A recently published study showed no direct association between skin biopsy findings and overall neuropathic pain (56). Another limitation to a broader use of IENFD measurements in the work-up of SFN is the inaccessibility of accredited laboratories performing the analysis.

#### *1.4.2.3 Other methods assessing small fiber neuropathy*

There are several other methods to evaluate SFN. Quantitative sudomotor axon reflex test evaluates the postganglionic sympathetic sudomotor function (16), whereas laser evoked potentials assess the nociceptive pathways. A CO<sub>2</sub> laser delivers heat pulses to the skin and excites free nerve endings in the superficial skin layers. It generates a pain sensation which is transmitted and recorded in the CNS (57, 58). Sudoscan is a non-invasive assessment of autonomic neuropathy, based on the electrochemical reaction between sweat chlorides and stainless-steel electrodes on hands and feet, and has been shown useful in an early diagnosis of PNP in hATTR amyloidosis (59), in Fabry Disease (FD) (60) and in diabetic neuropathy (61). Pain-related evoked potentials, a non-invasive method using concentric electrodes stimulating A-delta fibers in superficial layers in dermis, has been shown to be a marker of SFN impairment in many SFN-associated conditions such as diabetes, HIV, FD and even in mixed neuropathies as CIDP, vasculitic neuropathy and CIAP (62-66). Yet another method is corneal confocal microscopy, a non-invasive method assessing reduction of corneal nerve density and sensation, which correlates with reduced IENFD in patients with SFN and predates other signs of neuropathy in diabetic patients (67-71). Further, detection of small

fiber damage by corneal confocal microscopy has been assessed in various neuropathies, such as idiopathic SFN, CIDP, and CMT1A (70, 72, 73).

## **1.5 ETIOLOGY OF POLYNEUROPATHY**

The etiology of PNP varies from systemic disorders, infections, hereditary, inflammatory, toxic to vitamin deficiencies etc. The numerous etiological causes pose a challenge to the neurologist seeing a patient with PNP, since the etiological investigation for each patient needs to be individualized depending on the clinical and electrophysiological phenotype of the PNP. A further challenge is to know when to stop searching for a cause and conclude that the PNP is idiopathic.

The most common identifiable cause of PNP is diabetes mellitus. Other common causes are toxic, immune-mediated and hereditary (10, 12). About one fourth of the patients have an idiopathic PNP (10, 12). Vitamin-B12 deficiency, a frequently cited cause of PNP, is found in only 3-4% of patients with PNP (10, 12).

### **1.5.1 Etiology of axonal polyneuropathy**

In patients with large fiber neuropathy without an underlying diabetes mellitus, idiopathic PNP is much more likely to be present in the subgroup with axonal rather than demyelinating sensorimotor neuropathy, since the latter subtype of neuropathy is more often due to inflammatory or hereditary causes.

Toxic neuropathy, either due to drugs (most common chemotherapy-induced) or alcohol, is often axonal (74, 75). It is suggested that the ethanol toxicity causes a slowly progressive sensory predominant small fiber affection, while the neuropathy due to thiamin deficiency is mainly a large fiber neuropathy with motor dysfunction (74).

As earlier mentioned, the classical PNP due to Sjögren's syndrome or malignancy is a strict sensory axonal PNP, either LD or NLD i.e. neuronopathy, often causing severe sensory ataxia. However, other phenotypes are described including both sensorimotor large fiber neuropathy and pure SFN (28, 29, 76).

Immune-mediated axonal neuropathy is usually due to an underlying vasculitic process. This can be a part of a primary systemic vasculitis or a non-systemic vasculitis restricted to the PNS, though isolated PNS vasculitis may sometimes affect the vasculature in the muscles as well (27). The phenotypes of vasculitic neuropathy include mononeuritis multiplex 45%, asymmetrical PNP 35% and symmetric distal PNP 25% (27).

#### *1.5.1.1 Hereditary axonal polyneuropathy*

The hereditary axonal PNP, which is also known as CMT2 (currently classified from CMT2A to CMT2Z), has both autosomal dominant and recessive forms (<http://neuromuscular.wustl.edu/time/hmsn.html>). The severity in phenotypes differs and age of onset is usually in childhood or in the first two decades, though onset as late as the seventh

decade has also been described (77). Most of the variants of CMT2 are rare, with the exception of CMT2A, which accounts for about 20% of the axonal CMT (77, 78). CMT2A is caused by mutations in the gene, *MFN2*, encoding the mitochondrial protein, mitofusin 2 (MFN2), and the phenotype is often severe and with co-existing CNS affection and optic nerve atrophy (77).

#### *1.5.1.2 Hereditary ATTR amyloidosis*

Another hereditary axonal PNP is hATTR amyloidosis, in which FAP is one of the phenotypes. Hereditary ATTR amyloidosis is caused by mutations in the gene encoding for transthyretin (TTR). The mutated TTR tetramer is instable and aggregates, leading to deposition of amyloid fibrils in vulnerable organs such as peripheral nerves (43, 79). There are more than 100 point mutations identified in the *TTR* gene, with the most common one being Val30Met (79, 80). Hereditary ATTR amyloidosis is endemic in the north of Sweden, and Val30Met constitutes at least 95% of the Swedish *TTR* mutations (81). In Sweden the hATTR Val30Met amyloidosis has a late-onset. Symptoms seldom start before the age of 50-60 years (79). The penetrance is low and age-dependent, rising from 10% among 50 year-olds to 70% among 90 year-olds (81). In contrary, the early-onset (symptoms starting before the age of 50) hATTR Val30Met in endemic areas in Portugal and Japan has a high penetrance and the PNP phenotype has a more autonomic profile compared to the late-onset form in Sweden (79). In general, the PNP phenotypes of hATTR amyloidosis include both LD-SFN with severe autonomic symptoms and axonal large fiber neuropathy with progressive walking disability (43). In non-endemic areas hATTR amyloidosis is under-diagnosed (43). There is now available pharmaceutical treatment for hATTR amyloidosis, with even a wider range of treatment options available in a close future. For many years removing the source of mutated TTR by a liver transplant was the only available treatment for hATTR, though with variable results (80). Since about five years a TTR stabilizer, tafamidis, is available for treatment of hATTR amyloidosis in patients with PNP stadium 1 (patients being able to walk without aid), and tafamidis has been shown to provide a long-term delay of neurological progression and delayed decline in nutritional status as well (82). Another effective TTR stabilizer is diflunisal, a NSAID agent (83). Just recently, two different phase 3 clinical trials with *TTR* gene silencing as treatment approach, were completed. Very promising results were presented at the first European ATTR amyloidosis meeting in November 2017 in Paris but the studies are not yet published.

Considering that hATTR amyloidosis is under-diagnosed in at least non-endemic areas, and the available and up-coming treatment possibilities, the usefulness of screening for hATTR amyloidosis among patients with idiopathic SFN is a relevant question. Levine et al. screened 47 US patients with idiopathic SFN with or without large fiber affection and found no amyloidogenic mutations in the *TTR* gene (84). Just recently a Chinese study identified three cases of hATTR amyloidosis (all Ala97Ser) in a cohort with 100 idiopathic pure SFN patients (85).

*When we started our screening study of hATTR in patients with idiopathic SFN (Study IV) there were no published screening studies of the TTR gene in patients with idiopathic SFN.*

*The US study was published (84) during our on-going work with the manuscript, and the second screening study was published (85) soon after our first submission.*

### **1.5.2 Etiology of small fiber neuropathy**

In SFN the described different etiologies are even more numerous, with diabetes mellitus being the most common one reported (16, 20). Other possible etiologies or co-morbid associated conditions include IGT (86, 87), connective tissue disease (88, 89), sarcoidosis (90), celiac disease (91, 92), thyroid dysfunction (34, 93), vitamin B12 deficiency (34), monoclonal gammopathy of underdetermined significance (MGUS) (24, 94), HIV and hepatitis C infections (95, 96), amyloidosis (43), toxicity due to alcohol or drugs (74, 97), paraneoplastic (28), fibromyalgia (98), mitochondrial disease (MD) (99) and hereditary neuropathies (100).

A step forward in the search for etiology of SFN was taken by Faber et al. in their publications of gain-of-function mutations in sodium channels as a cause of hereditary SFN (101-103). In 29 % of 28 patients with idiopathic pure SFN new missense mutations in Nav1.7 were identified (101). Later on, the same group reported mutations in Nav1.8 and Nav1.9 in patients with painful idiopathic predominant SFN lacking mutations in Nav1.7 (102, 103). A prevalence of 16.7% of sodium channel gene mutations was found in a cohort of 921 patients with pure SFN (25). Faber and colleagues have now an on-going clinical trial with treatment with lacosamide in patients with gain-of function Nav1.7 mutations (104).

### **1.5.3 Fabry disease as a cause of small fiber neuropathy**

Fabry disease is nowadays a treatable (with enzyme-replacement therapy ) X-linked lysosomal storage disorder due to a partial or complete deficiency of the enzyme  $\alpha$ -galactosidase A ( $\alpha$ -GAL), resulting in accumulation of glycosphingolipids in different organs (105). More than 600 mutations are described in the  $\alpha$ -galactosidase A (*GLA*) gene that encodes  $\alpha$ -GAL (106). Males are usually more severely affected with a classical phenotype, but heterozygous women may have disease manifestations (107-110). Symptoms from involvement of the peripheral nerves are common (107, 108, 111). The estimated incidence of FD is 1 in 40-60,000 males (or 1 in 117,000 in the whole population) (112, 113). The idea of FD as an underdiagnosed disease in the general population arose with the publication of a screening study of new-born boys in Italy (113). Spada et al. found an incidence of 1 in ~3100 new-born boys, and a higher frequency than expected of so-called late-onset mutations (11:1; late-onset:classical phenotype) (113). The initial symptoms of late-onset FD are usually from an isolated organ system. Screening studies of patients with cryptogenic stroke, hypertrophic cardiac disease or renal disease have shown a higher frequency of FD than expected (0,3-0,5%) (114-116).

Small fiber neuropathy with a predominant A $\delta$  fiber affection is the main manifestation of FD in the PNS (66, 107, 117-119), though mild large fiber axonal sensorimotor neuropathy may also occur (117). The dominant symptom is neuropathic pain, which can be chronic, distributed as a classical LD-SFN and/or acute pain in the extremities, so-called Fabry crises

(120, 121). The crises are triggered by rapid changes in body temperature and manifest themselves in situations like fever, physical activities and hot weather.

#### *1.5.3.1 Biomarkers in Fabry Disease*

The enzyme activity of  $\alpha$ -GAL is often reduced or absent in males with the classical phenotype (122). However, in late-onset FD or in females the  $\alpha$ -GAL activity may be within normal ranges. The globotriaosylceramide (Gb<sub>3</sub>), the substrate for  $\alpha$ -GAL, is measurable in plasma and urine, and especially an elevated urine-Gb<sub>3</sub> has been considered a hallmark for FD, however it harbors the same low sensitivity as a biomarker as  $\alpha$ -GAL in females (122). A product of the Gb<sub>3</sub> deacylation, globotriaosylsphingosine (lyso-Gb<sub>3</sub>) is currently the golden standard biomarker for FD (122). Lyso-Gb<sub>3</sub> has also been suggested as an effective biomarker for treatment response (122), as well as a relevant biomarker for determining the clinical relevance of *GLA* gene variants of unknown clinical significance (123).

#### *1.5.3.2 Genetic variants of unknown clinical significance*

There are several both exonic and intronic variants in the *GLA* gene, whose pathogenicity are under debate.

The D313Y alteration was found in 0.45% of subjects in a normal Caucasian population (124). The same group also showed that the D313Y enzyme variant was stable in lysosomal pH (4.5), but the enzymatic activity decreased in neutral pH (124). This indicates that even if the measured  $\alpha$ -GAL activity is reduced, the enzyme still is active in the lysosomes.

However, D313Y has been found as the single genetic alteration in patients with highly reduced  $\alpha$ -GAL activity and end-stage renal disease (125), in stroke patients (126), and in patients with cardiovascular disease (127). D313Y has been suggested to be associated with white matter lesions (128, 129). Just recently, du Moulin et al. published a case series of 14 German patients with D313Y (130). Ten patients had symptoms and clinical manifestations of FD, most common were neurological (stroke and pain) and ocular manifestations (130). The authors suggest annual clinical evaluations and in symptomatic patients' treatment can be considered (130). Similarly, in a Greek cohort of 17 D313Y patients; five patients fulfilled the definite FD criteria, eight patients had some symptoms of FD, two had no FD signs and two were healthy (131). All patients had normal lyso-Gb<sub>3</sub> activity while urin-Gb<sub>3</sub> and  $\alpha$ -GAL activity varied (131).

On the contrary, Oder et al. did not identify any severe FD manifestations such as pain, pain crises or cardiac involvement in five D313Y patients (132). The biochemistry markers were also normal. One patient had white matter lesions and one had a transitory ischemic attack (TIA). The patients were stable during a four-year follow-up (132). Finally, the D313Y variant was not considered pathogenic in two Danish families or in a case report of two German patients (133, 134).

The R118C variant was first identified by Spada et al. as a novel possible disease-causing late-onset mutation (113). It has been proposed as pathogenic in patients with chronic kidney



failure (115, 125) and cryptogenic stroke (126, 135, 136). However, just recently a retrospective evaluation of clinical, biochemical and histopathological data of 22 patients with R118C showed no shortened life time expectancy or major organ complications (137). Some patients had angiokeratoma. The authors suggest the R118C variant to be of low-pathogenicity or non-pathogenic and not to motivate enzyme replacement treatment (137).

There are also intron variants whose pathogenicity are debated, such as the cardiac variant IVS4+919G>A [rs199473684] (138, 139) and the haplotype IVS0-10C>T [rs2071225] IVS2-81\_-77delCAGCC [rs5903184], IVS4-16A>G [rs2071397], IVS6-22C>T [rs2071228] (*referred as haplotype 1 in Study I*), which is suggested to cause neurological manifestations (140-142).

#### *1.5.3.3 Screening studies in patients with small fiber neuropathy*

Considering that FD might be underdiagnosed as well as treatable and the hypothesis that isolated organ manifestations are seen in late-onset FD, screening studies for FD in patients with SFN have been performed (85, 141, 143).

Tanislav et al. identified one case of FD and four patients with the intronic haplotype 10C>T mentioned above, in the *GLA* gene in combination with pathological biomarkers, in a small cohort of 24 German patients with idiopathic SFN (141). This was not confirmed in a large genetic screening study of 440 Dutch patients with isolated SFN (143), where no cases of FD were identified. The authors reported a R118C variant in a female patient, but she had normal biomarkers and no typical FD pain (143). In a recently published Chinese study of 100 patients with isolated idiopathic SFN, one patient who was identified through screening with Dried Blood Spot  $\alpha$ -GAL activity, was later confirmed to have FD by genetic investigation (85).

*When we began planning our first study (Study I) there were no earlier genetic screening studies for FD in idiopathic SFN. During our on-going work the first German pilot study was published (141). The novelty of our study was however, that it was the first controlled study. Still, very few patients were included in the above-mentioned studies, as truly idiopathic SFN patients are hard to find. So when the opportunity for a larger study appeared through a Nordic collaboration, we found it interesting to be a part of that project. Ironically, in the course of the Nordic SFN study (Study IV), the Dutch group published their large study of over 400 patients with SFN (however not just idiopathic cases).*

## **1.6 IDIOPATHIC POLYNEUROPATHY**

Different terms are used to describe this disorder in the literature: PNP of undetermined cause (144), chronic cryptogenic sensory PNP (145, 146), cryptogenic PNP (147), sensory-predominant painful idiopathic neuropathy (148), chronic idiopathic axonal polyneuropathy (CIAP) (6, 41, 149-161), cryptogenic axonal PNP (12, 162), idiopathic small fiber neuropathy (101), idiopathic distal small fiber neuropathy (163).

Here I have chosen to use the terms idiopathic SFN to represent a predominant or pure small fiber condition, and CIAP in accordance with current literature, to represent idiopathic axonal predominantly large fiber neuropathy.

### **1.6.1 Idiopathic small fiber neuropathy**

The percentage of patients with idiopathic SFN varies in the literature between 23% and 93% (18-21, 24, 25). The differences in the proportion of idiopathic SFN can partly be explained by the variance of the diagnostic set up for SFN and the quality of the etiological evaluation at primary, secondary, and tertiary (neuromuscular specialist center) centers.

*In our first study the percentage of idiopathic SFN diagnoses after review of referral documents and medical records was 25%, probably corresponding to the results of etiological investigations at the primary/secondary care centers. The percentage of patients with idiopathic SFN among the group that underwent our standardized focused investigation was 73%. This could represent the proportion of patients with idiopathic SFN in the total population of patients with SFN investigated at a tertiary center.*

Small fiber neuropathy, regardless of it being idiopathic or due to an identified cause, has a negative impact of quality of life mainly due to pain (1).

#### *1.6.1.1 A possible immunological underlying cause of small fiber neuropathy*

A current hypothesis about the pathogenesis of idiopathic SFN, at least in some patients, is immunological. Several immune-mediated diseases such as Sjögren's syndrome and paraneoplastic disorder, are associated with SFN (28, 88-90). Autoantibodies have been described in patients with SFN (164-166). Inflammatory infiltrates have been reported in sural nerves from patients with sensory dominant painful idiopathic neuropathy (148). Gene expression of proinflammatory cytokines are elevated in skin biopsies in patients with LD-SFN (167). Inflammatory cell infiltrates were identified, but did not correlate with cytokine levels (167). The same German research group has further showed elevated IL-10 and IL-6 gene expression in the sural nerve in painful neuropathies (168). In skin biopsies, regardless of neuropathy type (axonal, demyelinating, SFN, painful, inflammatory), the number of vessel-bound T-cells and macrophages was elevated, IL-10 and IL-6 were upregulated, while neurotrophic factors were downregulated compared to healthy controls (168, 169). An aberrant expression of inflammatory regulating microRNA in white blood cells, sural nerves and skin biopsies was recently reported in painful neuropathies (170).

Cases of SFN responsive to immunological treatment have been reported (171, 172). There is an ongoing randomized double-blinded placebo-controlled clinical trial with treatment with IVIG for idiopathic SFN, with pain relief as the primary outcome (173).

### *1.6.1.2 The chances of finding an etiology in patients with seemingly idiopathic small fiber neuropathy*

Devigili et al. could identify a possible cause in seven of 28 patients (25 %) with idiopathic SFN at a 2-year follow-up: two had IGT, four had diabetes mellitus, and one had Sjögren's syndrome (21).

Farhad et al. reviewed the charts of almost 300 patients with "idiopathic neuropathy", referred and reevaluated at a neuropathy center, consisting of pure SFN, mixed neuropathy and predominantly large fiber neuropathy (174). Thirty-three percent remained idiopathic, with pure SFN being the most common type of neuropathy with no identifiable cause. The most common etiology found was IGT (25.4%), in which diabetes was diagnosed in 9.2%, followed by CIDP (20%), MGUS (7%) and toxic causes (5.7%) (174).

In a recently published study, a standardized comprehensive diagnostic algorithm was performed in over 900 patients with pure SFN (143). The authors identified an additional associated condition in 26.7% (most frequent: sodium channel gene variants (13.8%), IGT (11.4%) or abnormal immunological laboratory findings (5.8%)) of the patients already known to have an associated cause of their SFN (143). The authors therefore suggest additional screening in patients already known to have a SFN associated cause, at least searching for autoimmune diseases, sodium channel gene mutations, diabetes mellitus including IGT, and vitamin B12 deficiency (143).

### **1.6.2 Chronic idiopathic axonal polyneuropathy**

Approximately 25% of patients with PNP have a CIAP (10, 12). The incidence of CIAP increases with age (12) and a male predominance has been reported (12, 146, 147, 152, 153).

Chronic idiopathic axonal polyneuropathy typically presents in the sixth decade with an insidious onset with symmetrical predominantly sensory or sensorimotor symptoms in the feet in a LD pattern with a slow progression rate (144, 146, 147, 152, 153). About 45% of CIAP patients develop symptoms from the hands (160). In general, CIAP is reported to be a relatively benign condition with limited motor impairment (146, 147) even after a 3-5-year follow-up (155, 175). However, Vrancken et al. reported that CIAP patients with an early onset (before age 65) have a more rapid progression rate and a higher grade of disability, than CIAP patients with a later onset despite the same disease duration (159).

Patients with CIAP may have small fiber involvement (149). Erdmann et al. reported neuropathic pain in 30 % of patients with CIAP (151). Patients with CIAP have impaired quality of life (3, 6, 151, 159). Vitamin B6 supplements, other nutrients or energy intake are not risk factors for CIAP (176, 177).

A recent updated Cochrane review of drug therapy for CIAP states that there are still no adequate randomized or quasi-randomized controlled studies published, and hence no recommended treatment for CIAP (178).

#### *1.6.2.1 The benefits of reevaluating the patients with chronic idiopathic axonal polyneuropathy*

Notermans et al. followed 75 patients with CIAP for five years. A definite etiology was found in only four patients (2 patients with CMT2 based on siblings developing symptoms, one with CIDP, one with alcohol abuse) (155). Sachedina et al. identified a possible etiology in only 3% (all IGT) upon reevaluation three years from baseline in a Canadian cohort of 228 idiopathic PNP patients (175). On the contrary, Rosenberg et al. reported that the diagnosis of idiopathic PNP was altered in as many as 49 % of the patients with CIAP after reevaluation (156). Misclassification were due to missed diagnosis of hereditary PNP, misinterpretation of clinical examination and test results as well as and not having followed the diagnostic guidelines (156).

The patients with CIAP are a heterogeneous group, partly due to the challenge in differentiating sporadic CMT2 from CIAP. Teunissen et al. compared 48 CIAP and 47 CMT2 patients clinically and electrophysiologically (179). CMT2 was found to start as a predominant motor axonal PNP and the patients had a larger extent of muscle atrophy, skeletal abnormalities and decline in electrophysiology parameters compared to CIAP patients. This was regardless of onset before or after 40 years of age in CMT patients. The authors concluded that in a patient with late onset, no family history and a predominant sensory or sensorimotor PNP, there is no need to search for a hereditary PNP (179). However, this was before the genetic “revolution”, nor were the CMT2 patients defined genetically. Still, the chance of identifying a genetic diagnosis, apart from the somewhat prevalent CMT2A, is minimal in older patients with a sporadic axonal sensorimotor PNP and not considered of any value in clinical practice (78, 180). However, this attitude might change now as gene panels and whole exon/genome sequencing are becoming more common and cheaper (181).

## **1.7 POSSIBLE HYPOTHESES FOR THE GENESIS OF IDIOPATHIC POLYNEUROPATHY**

### **1.7.1 Microangiopathy - a pathogenic cause of chronic idiopathic axonal polyneuropathy?**

#### *1.7.1.1 Associations between chronic idiopathic axonal polyneuropathy and the metabolic syndrome*

The incidence of CIAP increases with age (12). Several authors have demonstrated an association between the metabolic syndrome and CIAP, although the metabolic risk factor in question varies (20, 86, 87, 152, 158, 161, 182-186). However, Hube et al. detected no overrepresentation of the metabolic syndrome in CIAP patients compared to healthy controls (187).

In a study by Visser et al. the metabolic syndrome was more common in patients with CIAP (55%) compared to controls (34%), and even more frequent in patients with a painful predominantly sensory CIAP (62%) (158). The significant risk factors were abdominal obesity and hypertension (158). A prospective Italian study reported an increased risk of developing CIAP in patients with peripheral vascular disease (182). Lipid abnormalities have been shown to be an independent risk factor for idiopathic PNP in some studies (20, 152, 183, 186, 187), but not in others (188). Small studies with statin treatment in diabetic PNP have shown limited effect (189, 190). Statin use by itself has earlier been postulated as a risk factor for idiopathic PNP (191), but a large validated case-control study in Denmark found no association between statin use and idiopathic PNP (192). In a recent large prospective cohort study in the Netherlands, including more than 1000 patients, PNP was associated to the metabolic syndrome regardless of co-existing diabetes or not (186). The correlation got stronger with more components of the metabolic syndrome present. In sub-analysis waist circumference and triglycerides were identified as separate risk factors (186). The same research group further showed that obesity and hypertension are associated with a decline in peripheral nerve function in participants without symptoms and signs of PNP (193). Diabetes mellitus was as expected strongly associated to PNP, however impaired fasting glucose was not (186). In this study oral glucose tolerance test (OGTT) was not performed, hence the study does not contribute to the unanswered question below (186).

The role of IGT as an etiology of idiopathic PNP is still debated. However, several studies, including one controlled study (161), have indicated that IGT is a risk factor for neuropathy (20, 86, 87, 161, 184, 185, 194, 195). On the other hand, Hughes et al. could not confirm IGT as a risk factor for CIAP in a controlled study (152), and an increased prevalence of large fiber neuropathy or SFN was not detected among patients with impaired glycemic control compared to healthy controls (196, 197). In a Swedish study the authors found no difference in sural nerve conductions, IENFD or thermal thresholds between subjects with normal glucose values and subjects with IGT (198). Smith et al. showed improved IENFD and sural nerve sensory amplitudes after lifestyle intervention in patients with IGT and neuropathy, thus supporting a link between IGT and CIAP (184). Furthermore, an increase in IENFD was described after supervised weekly exercise during one year in patients with diabetes without PNP (199).

Additional support for the link between CIAP and ischemia are the reports of a higher frequency of PNP in patients with obstructive sleep apnea and chronic obstructive pulmonary disease (COPD) (200-203). However, the latter was not confirmed in a large case-control study, in which the prevalence of COPD did not differ between CIAP patients and controls (162).

#### *1.7.1.2 Microangiopathy in endoneurial microvessels in sural nerve biopsy*

One possible cause of CIAP is disturbed microcirculation in the vasa nervorum in the PNS. Several histopathological studies of sural nerves from patients with diabetic neuropathy have shown microangiopathic abnormalities in endoneurial microvessels (204-215). The most

persistent finding of microangiopathy yet reported is an increased basal area thickness or basal membrane area (204, 205, 207, 209-212, 214, 215). Other main parameters suggestive of microangiopathy are endothelial hypertrophy, i.e. increased endothelial area (204, 209, 211, 215, 216), endothelial hyperplasia, i.e. increased number of endothelial nuclei per vessel (204, 206, 209, 215), increased endothelial profile number (209-212), and decreased lumen area (210, 211). A frequent qualitative description is an increased reduplication of the basal laminae (204, 208, 215). Microvessel abnormalities correlate with the severity of the neuropathy (206, 208, 209, 215) and microangiopathic changes are reported in diabetic patients with both mild and subclinical neuropathy (208, 210, 212).

Similar microangiopathic changes have been shown in endoneurial microvessels in sural nerves from patients with atherosclerotic peripheral vascular disease and patients with COPD (217, 218).

Considering the clinical and electrophysiological phenotype resemblances of CIAP and diabetic neuropathy, one would expect comparable histopathological findings in endoneurial microvessels in patients with CIAP. This was indicated in an ultrastructural study of Teunissen et al. in year 2000, in which they compared microangiopathic parameters in sural nerves from 18 CIAP patients with three different control groups; HMSN 2 (n=6), autopsy controls (n=10) as negative controls and diabetic neuropathy (n=4) as positive controls (219). The basal lamina area thickness (BLAT) and the endothelial cell area (EA) were in the same range in CIAP and diabetic neuropathy. The BLAT was significantly larger in CIAP patients in comparison with HMSN 2, but not compared to autopsy controls ( $p=0.08$ ) (219). The EA was increased in CIAP compared to HMSN 2. In autopsy cases the EA was enlarged compared to all the other groups, probably due to post mortem changes (219). In concordance, Hube et al. found that the basement membrane thickness and the number of endothelial cell nuclei in endoneurial microvessels in sural nerve biopsies from ten CIAP patients and seven patients with diabetic neuropathy did not differ significantly, hence the authors concluded that microangiopathy was present in CIAP patients (187). However, this was not an ultrastructural study, the analyses were performed in light microscopy and a negative control group was lacking (187).

*For many years the only study assessing microangiopathy in CIAP patients was the study by Teunissen et al. published in year 2000 (219). So our biopsy study (Study II) started out as a confirmative study of the single preexisting study. Study II was published in year 2016 and the third study addressing the same hypothesis was published in 2017 (187).*

#### *1.7.1.3 Possible association between microangiopathy in the peripheral and the central nervous system*

In the CNS, microangiopathy leads to white matter changes (WMC), which is an independent risk factor for developing vascular cognitive impairment and vascular dementia (VD) (220, 221). Metabolic risk factors, such as diabetes and hypertension, are associated with an increased risk of WMC (222, 223), vascular cognitive decline (221, 224) and Alzheimer's

disease (AD) (associated with diabetes) (225). There is one small hospital-based study reporting high comorbidity between PNP, dementia and vascular risk factors (226).

Just recently, Ferik et al. addressed the question whether there is an association between PNP and WMC in patients with diabetes mellitus type 2 (227). Sixty-six patients with diabetes were included, mean age 54.5 years, and 60% of the patients had diabetic neuropathy (sensorimotor PNP). Patients with metabolic risk factors, except for dyslipidaemia, or other co-morbidities known to cause PNP were excluded. The patients with PNP had significant more WMC compared to patients without PNP, even after adjusting for age and duration of diabetes (227). Furthermore, there was no significant difference between the patients with or without PNP in regard to the presence of carotid atherosclerosis (227). This supports the idea of a common microangiopathic pathogenesis in the PNS and CNS.

*A step forward to investigate a potential association between microangiopathy in the PNS and CNS, clinically manifested as CIAP and vascular dementia, is taken in our Study III.*

### **1.7.2 Neurodegeneration as an alternative hypothesis for the pathogenesis of chronic idiopathic axonal polyneuropathy**

An alternative mechanism for the pathogenesis of CIAP is neurodegeneration causing disturbed axonal structure or function. The cytoskeleton of the neuron consists of three main groups of proteins: actin, microtubule and neurofilament. The subunit neurofilament light (NFL) is mainly expressed in large myelinated axons and elevated NFL levels in the cerebral spinal fluid (CSF) are well documented in several neurodegenerative conditions in the CNS, such as frontotemporal lobe dementia, VD, amyotrophic lateral sclerosis (ALS) (228, 229), but is also detected in other predominant subcortical diseases such as MS (230). In ALS and MS, CSF-NFL levels correlate with serum NFL levels (228, 230), but at least in MS the elevated serum levels are secondary to CNS damage.

Hence could serum/plasma NFL work as an axonal degenerative marker in PNS diseases? In Guillain-Barré syndrome significant elevated NFL levels were seen in both CSF and serum compared to controls (231). Though, that could still be a proximal root affection in the entrance of the spinal cord with a secondary leakage to plasma. However, elevated plasma NFL levels are detected in CMT patients compared to healthy controls (Abstracts, 2017 Peripheral Nerve Society Meeting July 8–12, 2017 Sitges, Barcelona, Spain, DOI 10.1111/jns.12225). The NFL levels correlate to the severity of the neuropathy. So elevated NFL in blood is an up-coming marker for peripheral axonal degeneration.

A clinical example where disrupted cytoskeleton leads to PNP is CMT2E, which is caused by mutations in the NFL chain gene (232).

#### **1.7.2.1 Autophagy**

Autophagy is the physiological cleaning process in the cells, in which damaged organelles and macromolecules are cleansed. In macroautophagy the membranes in the cell assemble and create a phagophore that internalizes damaged cell parts and forms a double-membrane

vesicle, an autophagosome. The autophagosome fuses with a lysosome and becomes an autolysosome, in which the degradation process occurs. This cleaning pathway is a normal process in the cell and has a critical role in the cellular homeostasis.

Dysfunction in the autophagy machinery has been suggested to be involved in the pathogenesis in several neurodegenerative conditions in the CNS, such as Parkinson's disease, Huntington's disease, AD and ALS, leading to aggregations of misfolded proteins (233). Furthermore, mutations in autophagy-related proteins cause muscle diseases. Danon disease, an X-linked disorder causing myopathy, hypertrophic cardiomyopathy and cognitive impairment, is caused by mutations in the *LAMP2* gene, which encodes for lysosome-associated membrane protein 2 (LAMP-2) (234). LAMP-2 is required for maturation of autophagosomes, when fused with lysosomes (234). Mutations in valosin-containing protein (VCP) cause Hereditary Inclusion Body Myositis with Paget's disease (235). Reduced VCP activity impairs the autophagy pathway and leads to accumulation of non-degraded autophagosomes (235). Other muscle disorders due to impaired autophagy are Acid Maltase deficiency (Pompe's disease, Glycogen storage disorder 2) (236), sporadic inclusion body myositis (237), drug-induced autophagic vacuolar myopathies (238), and X-linked myopathy with excessive autophagy (239).

Whether the autophagy pathway is involved in the pathogenesis in peripheral nerve disorders is not yet thoroughly explored.

In rats with metabolic abnormalities, increased number of autophagy-related structures (ARS), as well as an up-regulated autophagy pathway, have been reported in degenerative and regenerative axons (240, 241). Autophagy has been suggested to have a protective role, in preventing neuropathy in a rat model with metabolic risk factors (240). In nerve injury autophagy structures are up-regulated by Schwann cells and contribute to the degradation of myelin proteins and lipids (242). Human neuroblastoma cells exposed to sera from patients with diabetes and neuropathy showed an increased rate of autophagy, however, the same phenomenon was not seen in the cells exposed to sera from diabetes patients without neuropathy (243).

In 2015 our collaborative colleagues in Linköping showed that the autophagy pathway exists in human peripheral nerve (244). They examined ultrastructural signs of autophagy in the posterior interosseous nerve in patients with and without diabetes, finding significantly higher number of ARS in patients with type 1 diabetes compared to patients with type 2 diabetes and a trend towards higher numbers compared to controls ( $p < 0.06$ ). The patients with type 1 diabetes all had neuropathy whereas the patients with type 2 diabetes varied from severe neuropathy to almost a normal picture similar to the controls (244). In a recent ultrastructural study, the authors found no difference in the density of ARS in sural nerve biopsies between patients with type 2 diabetes compared to subjects with normal glucose tolerance and IGT at baseline (216). At a 11-year follow-up, most of the ARS were found in Schwann cells of sural nerves in the patients with type 2 diabetes, with no ARS identified in the myelinated axons (216). In parallel, the myelinated fiber density had declined significantly. Whether this



implies inhibition of the autophagic pathway in the axons in diabetic neuropathy, or is merely an indication of the autophagic degradation process taking place in Schwann cells is not clear.

*Whether autophagy is present in sural nerves of CIAP patients is investigated in our biopsy study (Study II).*

### **1.7.3 Mitochondrial dysfunction - a possible role in the pathogenesis of polyneuropathy**

Polyneuropathy is common among patients with mitochondrial disorders with a reported frequency of 12-45% (245-247). Polyneuropathy exists as one of the affected organ systems in several mitochondrial syndromes such as MELAS, MERFF, LHON, KSS, MEO, Leigh syndrome, MNGIE, SANDO, MIRAS, MEMSA, AHS, IOSCA, ADOA (248). In NARP (Neuropathy; Ataxia and Retinitis Pigmentosa) an axonal predominant motor PNP is the dominant feature, and it is due to mutations in *MTATP6*, causing deficits in ATP production by complex V of the respiratory chain (249).

Polyneuropathy can also be a feature in a non-systemic MD and often present as asymmetric, mostly axonal, sensorimotor multifocal neuropathy (247, 248), although SFN has been reported as a single manifestation in the PNS in mitochondrial myopathy (99).

Mitochondria are actively transported in antero- and retrograde directions along the cytoskeleton in the peripheral nerve according to the local energy requirements. Their shape, length, number and size are controlled by mitochondrial fission and fusion, and balance between the two states is regulated by dynamin-related proteins. Dysfunctional mitochondria are removed by mitophagy. These mitochondrial dynamics are crucial for the energy requirements in peripheral axons (250).

As mentioned in section 1.5.1.1, CMT2A is due to mutations in the *MFN2* gene which encodes a large mitochondrial transmembrane GTPase responsible for mitochondrial fusion. Another CMT variant with mitochondrial involvement is CMT2K, which is due to mutations in ganglioside-induced differentiation-associated protein 1 (*GDAP1*) gene, which encodes a mitochondrial fission factor and is crucial for mitochondrial outer membrane targeting activity (248).

The primary protein involved in mitochondrial fission is Dynamin-related Protein 1 (Drp1) (251, 252). Increased mitochondrial fission is linked to neurodegeneration through the process of disturbed mitochondrial mitophagy (251). Drp1 is up-regulated in dorsal root ganglia of experimental models of diabetes secondary to hyperglycemia and Drp1 inhibition relieves neuropathic pain in diabetic rats (253, 254). Whether Drp1 is up-regulated in human peripheral nerves in diabetic neuropathies or CIAP is not known. Mitochondrial dysfunction has been suggested to be involved in the pathogenesis of diabetic neuropathy, but further studies on how the diabetic state affects the expression of mitochondrial transport and fusion/fission proteins are needed (255).

Mitochondrial dysfunction may also play a role in the pathogenesis of toxic neuropathies. Bobylev et al. showed loss of total mass and changed morphology of mitochondria in axons in a cisplatin-induced neuropathy in mice (256). The disturbed mitochondrial dynamics were partly due to reduced expression levels of the fusion protein MFN2 (256).

One can speculate if idiopathic PNP can be due to mitochondrial dysfunction, either as a consequence of energy depletion or disturbed mitochondrial dynamics along the peripheral nerve.

*In Study III a potential association between idiopathic PNP and MD on a population level is studied.*

To summarize, idiopathic PNP is a common neurological disorder with a significant morbidity and decreased quality of life. Since the etiological cause per definition is unknown there is no available curative or disease-modifying treatment. There is hence a need for further knowledge about possible etiological factors in SFN and to find the key behind the pathogenesis of CIAP.

## **2 AIMS**

My overall aim was to explore the possible etiologies of idiopathic PNP by probing idiopathic PNP from different angles, such as clinical phenotyping, electrophysiology, histopathology and to investigate potential hereditary causes and comorbidities.

### **2.1 SPECIFIC AIMS**

- To show the spectrum of etiological factors that could be identified in patients with seemingly idiopathic SFN, if a standardized focused investigation was applied (Study I).
- To investigate whether Fabry Disease could be a cause of idiopathic SFN with or without large fiber involvement in young to middle-aged patients (Study I).
- To explore the presence of microangiopathy in microvessels in sural nerve biopsies from patients with CIAP in comparison to controls without sensory PNP and patients with inflammatory neuropathy (Study II).
- To study whether the density of autophagy-related structures in the peripheral nerves of patients with CIAP differs from the density observed in the controls (Study II).
- To assess the risk of specific comorbid diseases with possible shared pathogenic mechanisms, i.e. mitochondrial disease and vascular dementia, in patients with CIAP (Study III).
- To further explore the value of genetic screening for Fabry Disease and hereditary ATTR amyloidosis in patients with idiopathic SFN with or without large fiber affection in a clinical setting in the Nordic region (Study IV).



### **3 MATERIAL**

The patient material in the first three studies in this thesis consisted mainly of patients from our outpatient clinic at the Department of Neurology at Karolinska University Hospital in Stockholm, whereas Study III was a register-based study based on national population data. The approach of recruiting patients was quite similar in Study I and IV, and almost all the patients were included during a visit at our outpatient clinic. Study II was a retrospective biopsy study, in which all biopsies were obtained from patients at the Department of Neurology.

#### **3.1 PATIENT POPULATION STUDY I AND IV**

The inclusion process of patients in Study I and IV consisted of a retrospective- and a prospective part. Patients older than 18 years with idiopathic SFN with or without large fiber sensorimotor axonal PNP (SFN or mixed neuropathy i.e. combined SFN and large fiber neuropathy) were recruited, though in Study I the upper age limit was 60 years.

If not otherwise stated throughout the thesis, the term idiopathic SFN in Study I and IV, includes both patients with isolated SFN and patients with mixed neuropathy.

Since Study IV was a multicenter study with nine different inclusion centers in four Nordic countries and the inclusion process somewhat varied between the different centers, the description in this section is restricted to the patients included in Sweden.

##### **3.1.1 Retrospective part**

The diagnosis register from February 2002 to April 2009 at the Department of Neurophysiology at Karolinska University Hospital was searched for the diagnosis code “small fiber neuropathy”. Primary care referrals to the Department of Neurophysiology as well as medical records at the Department of Neurology were reviewed, and patients with a possible etiology identified for SFN were excluded (Study I).

The same procedure was performed for Study IV from 2000 to 2015, however only medical records from patients found in both the diagnosis register from the Department of Neurophysiology at Karolinska University Hospital with the diagnosis code “small fiber neuropathy” and the diagnosis register from the Department of Neurology at Karolinska University Hospital outpatient clinic, ICD-10 codes G62.9, G60.3, G60.9 were reviewed. Medical records at the Department of Neurology were studied, and patients with a possible etiology identified for SFN were excluded.

At Uppsala University Hospital medical records from patients who visited the outpatient clinic at the Department of Neurology with the ICD-10 codes G62.9, G60.3 or G.60.9 during year 2000 to 2015, were reviewed (Study IV). If the patient had a pathological QST and no identified etiological cause the patient was approached for participation in the study.

### **3.1.2 Prospective part**

Patients were recruited from the Departments of Neurology and Neurophysiology at Karolinska University Hospital in Stockholm (Study I and IV), the University Hospital in Linköping (Study I), and through collaboration with private-practice neurology and neurophysiology departments in Stockholm (Study I) during the inclusion periods respectively (Study I: September 2007 and October 2009, Study IV: October 2015 - February 2017).

### **3.1.3 Inclusion process**

Here the two studies diverged. Since one of the exclusion criteria in Study IV was previous performed genotyping of the *GLA* and/or *TTR* genes, no patients from Study I were included in Study IV.

#### *3.1.3.1 Study I*

The remaining patients with seemingly idiopathic SFN were asked to participate in a clinical screening visit i.e. a standardized focused investigation, including an interview, clinical examination and an extensive laboratory investigation. After exclusion of other possible etiologies, the patients were invited to participate in the genetic part of the study screening for FD.

#### *3.1.3.2 Study IV*

In the retrospective part of the study, if the SFN had been deemed as idiopathic by the responsible neurologist at the time-point of SFN diagnosis, the patient was contacted by letter and asked to participate in the study. Thereafter followed a telephone interview to further establish the diagnosis of idiopathic SFN, and then a visit at our clinic for examination and blood samples. No further etiological investigation was performed. If potential SFN-associated co-morbidities were identified to have had an onset after the time-point of SFN diagnosis, a case-to case judgement regarding the possible association to SFN was performed.

For the prospective part, only patients with a negative etiological investigation (i.e. diabetes, uremia, thyroid disease, vitamin deficiency, immune mediated neuropathies, alcohol abuse, neurotoxic drugs and infections) were asked to participate in the study.

## **3.2 PATIENT POPULATION STUDY II**

In this retrospective study, fascicular sural nerve biopsies from ten patients with CIAP were used. Three patients (two women and one man) were included in both Study I and II.

The biopsies of patients with CIAP and the control group of patients with inflammatory neuropathy, see below, had been obtained by Dr. R. Press during the period 2002 – 2009 and had been stored in the Biobank at the Department of Clinical Pathology, Karolinska University Hospital, Huddinge.

### **3.3 PATIENT POPULATION STUDY III**

Study III was a strictly register-based study, in which we explored the risk of MD and VD in patients with CIAP and used AD as a dementia control condition for which we presumed little shared underlying mechanisms with CIAP.

#### **3.3.1 Study base**

All individuals born in Sweden and included in the 1990 Swedish Population and Housing Census were followed from January 1<sup>st</sup> 2001 to December 31<sup>st</sup> 2010 (N=7 792 012). To reassure ourselves that the polyneuropathies were truly idiopathic in nature, we excluded from the study base all individuals with comorbid diseases known to contribute to the pathogenesis of PNP (see Methods).

The study base was reduced to 5 535 955 individuals after these exclusions. For the included individuals, the follow-up stopped at their first hospital visit (diagnosis) for CIAP, death, migration out of Sweden, or December 31<sup>st</sup> 2010, whichever occurred first.

#### **3.3.2 Identifying CIAP patients and definitions of MD, VD and AD**

##### *3.3.2.1 The Swedish Patient Register*

The Swedish Patient Register has been collecting information on hospital discharge diagnosis since 1964/1965 (nationwide from 1987) and hospital-based specialist outpatient visits (but not primary health care) since 2001. All diagnoses in the register are coded according to the Swedish revisions of the International Classification of Diseases (ICD-7 until 1968, ICD-8 during 1969-1986, ICD-9 during 1987-1996 and ICD-10 since 1997).

##### *3.3.2.2 Identifying CIAP patients*

CIAP cases were identified in the Swedish Patient Register as all individuals with at least two hospital visits between January 1<sup>st</sup> 2001 and December 31<sup>st</sup> 2010 with the ICD-10 codes for unspecified polyneuropathy (G62.9) or idiopathic progressive polyneuropathy (G60.3). The date for the first hospital visit was defined as the date for the CIAP diagnosis, i.e. index date. All patients with any CIAP-related hospital visits before January 1<sup>st</sup> 2001 (ICD-7 364,99 and 364,1; ICD-8 354,09; ICD-9 356X, 356E and 356W; ICD-10 G60.3 and G62.9) were excluded.

##### *3.3.2.3 Definition of MD, VD and AD*

All individuals with MD, VD and AD were identified during 1987-2010 from the Swedish Patient Register (inpatient hospital visit during 1987-2000, and both inpatient and outpatient hospital visit from 2001 onward) using relevant ICD-9 and -10 codes, (which codes used is specified in the supplementary information table S1 in the manuscript).

### **3.4 CONTROLS**

#### **3.4.1 Controls for the genetic analysis in Study I**

203 age- and gender-matched healthy anonymous blood donors were used as controls for the genetic analysis in Study I. We have no further information regarding risk factors or other associated diseases among the controls, however known FD is an exclusion criterion for being a blood donor according to Swedish regulations. The selected patient:control ratio; 1:7, was arbitrary.

#### **3.4.2 Controls in the sural nerve biopsy study (Study II)**

Two age and gender-matched control groups were chosen for this study. One group consisted of eleven patients with inflammatory neuropathy and the other group contained ten normal controls without sensory PNP.

The biopsies from the normal controls had been obtained by Dr. Solders during the period 1981-1986. At that time-point at our neuromuscular center, sural nerve biopsies were performed in patients with symptoms of distal weakness and/or sensory symptoms from the lower limbs. The sural nerve biopsy was hence a routine procedure in an early disease state when suspecting PNP, as part of the clinical evaluation. The ten patients were later diagnosed with other conditions than sensory PNP. The biopsies have been used as normal controls in earlier publications (32, 257, 258).

The inflammatory neuropathy controls consisted of six patients with CIDP and five patients with isolated PNS vasculitic neuropathy. The patients had clinical and electrophysiological phenotype of either probable CIDP or isolated PNS vasculitic neuropathy. The results of the sural nerve biopsies further confirmed the diagnosis. No clinical evidence of other conditions than CIDP or of isolated PNS vasculitic neuropathy was seen upon long-term follow-up. The choice of inflammatory neuropathy as a control group was due to the relatively well-defined pathogenesis of their neuropathy.

#### **3.4.3 Controls in the register-based study (Study III)**

Incidence density sampling was used to randomly select five age- and sex-matched controls per CIAP case from the study base. The controls had to be alive and without CIAP diagnosis on the ascertainment date of their matched cases.

### **3.5 ETHICAL APPROVAL AND CONSENT**

All studies were approved by the regional ethical board in Stockholm. In Study I and IV all patients received oral and written information about the studies. Written informed consent was obtained from all patients' prior participation.



## **4 METHODS**

### **4.1 THE ASSESSMENT OF SMALL AND LARGE FIBER NEUROPATHY**

#### **4.1.1 Study I and IV**

The diagnosis of SFN was based on pathological results of QST according to a standardized method using the TSA II NeuroSensory Analyzer to determine detection thresholds for warm and cold sensations, and clinical examination indicating small fiber dysfunction in the extremities. Pathological findings of nerve conduction studies indicative of an axonal PNP were allowed, i.e. patients with mixed neuropathy.

#### **4.1.2 Study II**

The PNP had been verified with nerve conduction studies as a part of the clinical investigation in all CIAP patients and inflammatory controls at the Department of Neurophysiology at Karolinska University Hospital.

The density of myelinated and unmyelinated fibers was counted, see below.

### **4.2 DEFINITION OF THE IDIOPATHIC NATURE OF THE POLYNEUROPATHY**

#### **4.2.1 Standardized focused investigation in Study I**

This work-up consisted of an interview (determination of family history, medical history, past and present medications, exposure to environmental toxins, and alcohol drinking habits), a clinical examination and a comprehensive laboratory investigation (Table 2).

#### **4.2.2 Nerve biopsy study (Study II)**

Since this was a retrospective study of nerve biopsies obtained in a clinical setting a standard etiological procedure or clinical investigation of the patients was not performed. All ten patients had been examined at the neuromuscular center at the Neurology Department of Karolinska University Hospital, and with no underlying cause of neuropathy identified through an extensive interview and laboratory work-up. The biopsies were part of the etiologic investigation of patients with unknown cause for an axonal sensorimotor PNP. The use of the biopsy was intended to exclude isolated PNS vasculitis, which can have a clinical presentation as a symmetric sensorimotor axonal PNP (27), and amyloidosis.

#### **4.2.3 Nested case-control and cohort study (Study III)**

In this register-based study no review of the medical records to validate that the PNP was idiopathic, was performed. To reassure ourselves that our CIAP cases were truly idiopathic, all individuals with comorbid diseases known to be associated with PNP were excluded from the study base using ICD-7 to ICD-10 codes for the following conditions: diabetes mellitus type 1 and 2; malignancies, including cancer in situ and tumor of uncertain behaviour such as hematologic diseases (Polycythemia Vera, Myelodysplastic syndrome and MGUS);

alcoholism; thyroid diseases; rheumatologic diseases; psoriatic arthritis; inflammatory bowel disease; celiac disease; renal failure in the need for dialysis care; Hepatitis C; HIV; and cobalamin deficiency (for information which ICD codes used see supplementary information, Table S3, in the manuscript).

**Table 2. Laboratory investigation of patients with SFN in Study I**

<b>Disease association</b>	<b>Biochemical analyses</b>
<b>Routine basal blood samples</b>	Plasma C-reactive protein, sodium, potassium, creatinine, AST, ALT, albumin, and calcium; blood hemoglobin, platelets, leukocytes and sedimentation rate; serum thyroid-stimulating hormone
<b>Vitamin B12 or folic acid deficiency</b>	Serum cobalamin, blood folate/fasting-serum folate, plasma homocysteine, or serum methylmalonate
<b>Diabetes mellitus</b>	Glucose level and glycosylated hemoglobin level
<b>IGT</b>	Oral glucose tolerance test
<b>Alcohol abuse</b>	Serum percentage of Carbohydrate-Deficient Transferrin, plasma gamma-glutamyltransferase, or blood erythrocyte mean corpuscular volume
<b>Vasculitis</b>	Rheumatoid factor, antinuclear antibodies, anti-neutrophils, cytoplasmic antibodies, B-cells, complement factors, or hepatitis C
<b>Monoclonal gammopathies</b>	Serum protein electrophoresis
<b>Infections</b>	HIV, syphilis
<b>Celiac disease</b>	Transglutaminase antibodies
<b>Amyloidosis</b>	Abdominal fat biopsy performed in those with a symptom duration of <4 years, or if there were signs of large fiber involvement

#### **4.2.4 Nordic screening study of FD and hATTR amyloidosis in patients with idiopathic SFN (Study IV)**

In this study no standard etiological investigation protocol was used, as compared to Study I. In the retrospective part of this study we leaned on the clinical judgement of the investigative neurologist at the time-point of the diagnosis of idiopathic SFN. In the prospective part all patients were investigated according to the guidelines for etiological investigation in SFN at our clinic. The purpose of this approach compared to the standard focused investigation procedure used in Study I was to increase the relevancy for the neurologist day-to-day work in a clinical setting.

#### **4.3 GENETIC ANALYSIS**

In Study I Sanger sequencing of the *GLA* gene, including the exon–intron boundaries and parts of intron 4, was performed at the Albrecht-Kossel-Institute for Neuroregeneration, Faculty of Medicine, Rostock, Germany. In Study IV Sanger sequencing of the *GLA* gene exons 1-7 and part of intron 4 as well as *TTR* gene exons 1-4 was performed at the Research Program for Molecular Neurology, University of Helsinki, Finland. A multiplex ligation-probe assay (MLPA) was used to detect copy number variants (deletions or duplications) of the *GLA* gene in specific cases in Study I and in all cases in Study IV.

#### **4.4 BIOCHEMICAL ANALYSES FOR FABRY DISEASE**

In Study I the lyso-Gb<sub>3</sub> level in plasma was detected in all patients. In the six patients with genetic alterations in *GLA* of unknown clinical relevance the  $\alpha$ -GAL activity in blood leucocytes and the Gb<sub>3</sub> level in blood, as well as the total Gb<sub>3</sub> and Gb<sub>3</sub>-isoform *N*-tetracosanoyl (Gb<sub>3</sub>\_24) levels in urine were evaluated using mass spectrometry. A skin biopsy was taken from the gluteal region to detect possible Gb<sub>3</sub> deposits with immunohistochemistry and electron microscopy. All these analyses were performed in Rostock, Germany.

In Study IV analyses of  $\alpha$ -GAL and lyso-Gb<sub>3</sub> with Dried Blood Spot were performed at Karolinska University Hospital in the patient with the genetic variant R118C.

#### **4.5 SURAL NERVE BIOPSY ANALYSIS**

A fascicular sural nerve biopsy was performed in local anesthesia from the area posterior to the lateral malleolus. The numbers of obtained fascicles were one to nine. For detailed information about fixation and preparation, see Method Section Study II. All nerve biopsies had been previously analyzed by an experienced neuropathologist in a clinical setting.

The Epon embedded specimens were coded and mixed randomly by the laboratory assistant preparing the semi-thin sections prior to histopathological analysis. The slides were given a random number 1-31. The work at the light and electron microscope as well as the analysis

on micrographs were performed on coded specimens. The software program ImageJ (<http://imagej.nih.gov/ij/>) was used for all measurements and calculations.

#### **4.5.1 Parameters assessed in semi-thin sections (light microscopy)**

Digital images were taken at different magnifications (fascicle area (20x), identifying microvessels (40x) and counting and measuring myelinated fiber (60x)) by using a Nikon eclipse E600 light microscope equipped with a Nikon Digital Sight U1 camera.

##### *4.5.1.1 Capillary Density*

Direct calculation of endoneurial and subperineurial microvessels was performed from micrographs covering the total fascicular area from each sample. The density was calculated by dividing the numbers of vessels by the fascicle area (numbers/mm<sup>2</sup>).

##### *4.5.1.2 Myelinated Fiber Density and Diameter*

Myelinated fibers were counted in three randomly taken micrographs covering different areas and if possible from different fascicles. The density was assessed by dividing the total numbers of fibers from each sample with the area covered in the micrographs (numbers/mm<sup>2</sup>). For calculation of fiber diameter, each micrograph was divided in four quadrants. The perimeter of each fiber in the upper left quadrant in each micrograph was measured, and the diameters were calculated using ImageJ.

#### **4.5.2 Parameters assessed in ultra-thin sections (electron microscopy)**

For ultra-structural analysis a JEOL JEM-1200EX transmission electron microscope was used. The magnification for assessment in section 4.5.2.1-2 was 15,000x. For the parameters of microangiopathy 2,500-12,000x magnification adjusted to the vessel size was used, expect for counting the intra-endothelial junctions (8,000x-60,000x).

##### *4.5.2.1 Unmyelinated Fiber Density and Diameter*

In each sample 25 micrographs were prepared, and the numbers of fibers were counted and the diameter of each fiber was measured in each micrograph. Density of unmyelinated axons was calculated by dividing the number of axons with the area of the micrographs (number/mm<sup>2</sup>).

##### *4.5.2.2 Density of Autophagy-Related Structures*

In the 25 micrographs direct calculation of ARS was performed. The structures counted were dense osmophilic lysosomes, phagophores, autophagosomes, and autolysosome-like structures. Density of ARS was calculated.

##### *4.5.2.3 Parameters of Microangiopathy*

All endoneurial and subperineurial vessels identified in the EM sections were photographed. For exclusion criteria see Method Section in Study II. Circumferences of the lumen, the

endothelial cell layer and the total vessel including basal lamina were drawn on the micrographs and the areas were assessed by the software program (Figure 1). The area of the endothelial cell layer (EA) was calculated by subtracting the lumen area (LA) from the lumen and endothelial cell area (LEA). The basal membrane area (BA) also called the basal lamina area was calculated by subtracting the LEA from the total vessel area (VA). The basal lamina area thickness (BLAT) was calculated by subtraction of the radius of a circle equivalent of lumen and endothelial area (LEA), from the radius of a circle equivalent of the total vessel area (VA), ( $BLAT = \sqrt{VA/\pi} - \sqrt{LEA/\pi}$ ) (219). The numbers of the endothelial- and periendothelial cell nuclei and the endothelial cell profile (number intra-endothelial junctions) were directly counted in each vessel.

**Figure 1. Schematic drawing of assessment of microangiopathic parameters**

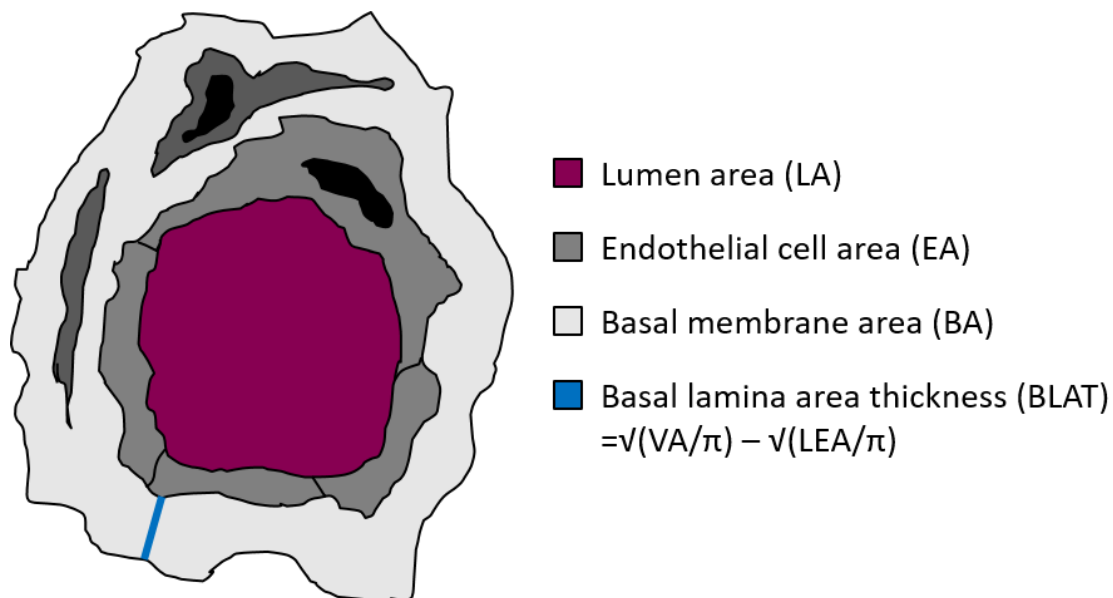


Figure 1. The basal lamina area thickness (BLAT) was calculated by subtraction of the radius of a circle equivalent of lumen and endothelial area (LEA), from the radius of a circle equivalent of the total vessel area (VA), ( $BLAT = \sqrt{VA/\pi} - \sqrt{LEA/\pi}$ ).

## 4.6 STUDY III

### 4.6.1 Nested case-control study

The nested case-control design implies the selection of a case-control sample from a parent cohort. All cases identified in the parent cohort during follow-up are included in the study sample. A pre-defined number of controls (usually not more than 5), free of the condition of interest, are randomly selected from the cohort and are usually individually time-matched to each case, as in our study. The use of a nested case-control study is suitable when the outcome of interest is rare and the parent cohort is large.

To study the subsequent risk of CIAP in relation to MD, VD, and AD, we performed a nested case-control study within the above-mentioned study base (3.3.1). A total of 2659 patients with CIAP were identified.

#### **4.6.2 Matched cohort study**

A cohort study enrolls a group of individuals, i.e. a cohort, and follows them for a defined time interval to ascertain an outcome of interest. In a matched cohort study for each exposed subject, a fixed number of unexposed subjects are selected, matched to the exposed on one or more confounders. When the exposure is rare, a matched cohort has the advantage of a smaller sample size compared to the full cohort, which facilitates the collection, management and analysis of detailed information.

We performed a matched cohort study, in which the cases (exposed) and controls (unexposed) enrolled in the nested case-control study were followed from the index date (i.e. date of the first CIAP diagnosis) to the first diagnosis of MD, VD, or AD, death, emigration, or end of 2010, whichever occurred first. In the matched cohort study we aimed to compare the subsequent risks of MD, VD, and AD between CIAP patients and individuals free of CIAP.

#### **4.6.3 Sensitivity analysis**

Patients with CIAP may only see the neurologist at a hospital-based clinic once to receive their diagnosis (one visit), and then have their follow-up visits at a primary care center. Because the Swedish Patient Register does not include information about primary care visits, including only patients with at least two hospital-based visits for CIAP in the analysis, might have led to a misclassification of some CIAP patients as individuals without this condition. To assess the influence of such definition on the study results, we conducted a sensitivity analysis where we included as well CIAP patients with only one hospital visit for CIAP during 2001-2010.

#### **4.6.4 Validation of definitions of vascular dementia and Alzheimer's disease**

The Swedish Drug Prescription Register, which collects data on all dispensed medications in Swedish pharmacies since 1 July 2005, was used to validate the specificity of the definitions of VD and AD. We extracted data from July 2005 to December 2010 and compared the frequency of prescribed drugs for AD (cholinesterase inhibitors (ATC code: N06DA02-4) and memantine (ATC code: N06DX01)) between the individuals with AD and VD. Sixty-six % of individuals with AD were prescribed drugs for AD, compared to 18% of the individuals with VD (Table S2 in the supplementary material in the manuscript).

Because there are currently no available drugs for prescription with the indication for MD in Sweden, we were not able to assess the definition of MD through prescribed drugs.

## **4.7 STATISTICAL ANALYSIS**

### **4.7.1 Study I, II and IV**

The Kruskal-Wallis multiple comparison test for non-parametric values was used for comparisons between groups (Study II) and countries (Study IV). For direct comparison between two groups Mann-Whitney U, two-tailed test for non-parametric values and unpaired t-test for comparing parametric values were used (Study I and II). Spearman's rank correlation was used for correlation analysis (Study II). Fisher's Exact test was used for contingency table (Study I and II). For the comparison of binary data between countries Chi-square test was used (Study IV).

In all studies the significance level was set to  $p < 0.05$ . Statistical analyses were performed using Graph Pad InStat Software (version 3.06) in Study I and Graph Pad Prism (version 6 and 7) (Graph Pad Software Inc, San Diego, USA) in Study II and IV.

### **4.7.2 Study III**

In the nested case-control study conditional logistic regression was used to calculate the odds ratios (ORs) with 95% confidence intervals (CIs) for the association of MD, VD and AD with the subsequent risk of CIAP.

Conditional Cox regression was used in the matched cohort study to estimate the hazard ratios (HRs) with 95% CIs for the association of CIAP with the subsequent risk of MD, VD and AD. The assumption of proportional hazards was assessed by introducing an interaction term between CIAP and time in the models. Separate Cox regression models for different time windows ( $\leq 1$ , 1-4,  $> 4$  years) after CIAP diagnosis were used to characterize how the HRs varied over time.

Potential effect modifications by sex and age were investigated through stratified analyses and by including interactions terms in the regression models for both the nested case-control and matched cohort studies.

For the statistical analyses SAS, version 9.4 (SAS Institute, Cary, NC) was used.

## 5 RESULTS

This section contains the main results from my four studies. More detailed and complete results are available in the published articles and manuscripts in the end of the thesis.

### 5.1 THE GENETIC SCREENING STUDIES (STUDY I AND IV)

As described in the Material and Method sections, the inclusion and exclusion processes in Study I and IV had both similarities and differences. The exclusion process led finally to 29 patients being included in Study I and 46 patients in Study IV (the Swedish cohort). These patients were genetically screened for FD (Study I and IV) and hATTR amyloidosis (Study IV), (Figure 2).

**Figure 2. Flowchart for inclusion and exclusion of patients in Study I and IV (Swedish cohort included in Stockholm and Uppsala)**

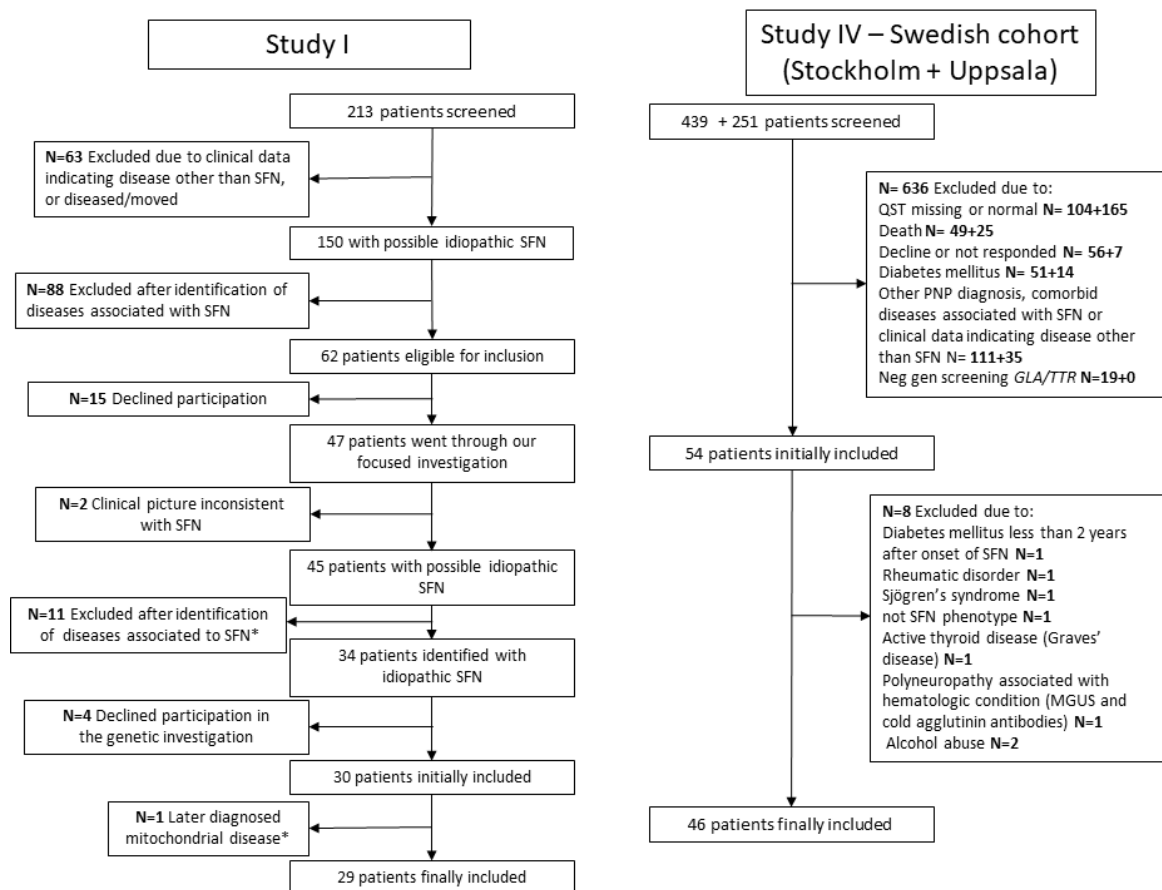


Figure 2. Inclusion and exclusion process in the genetic screening studies. \*The disease-associated comorbidities identified in the standard focused investigation are presented in Table 4.



### 5.1.1 Demographic and clinical characteristics

The patients in Study I were younger than the patients in Study IV, due to the different inclusion criterion, with an upper age limit of 60 years in Study I (Table 3). The older patient group might partly explain the longer duration of symptoms seen in Study IV. Study I had a female predominance ( $p=0.02$ ) and included more patients with an isolated SFN ( $p=0.15$ ). The difference in reported autonomic symptoms, might be due to that a more structured interview regarding autonomic symptoms was performed in Study IV. The most frequently reported autonomic symptoms in Study I were orthostatic hypotension, hyperhidrosis, erectile dysfunction, and problems related to micturition, while in Study IV the patients reported gastrointestinal symptoms, hypo- or hyperhidrosis, sicca symptoms and orthostatic hypotension.

**Table 3. Demographics and clinical characteristics of included patients**

	Study I n=29			Study IV n=46			
	Mean	Median	Range	Mean	Median	Range	p-value
<b>Age (years)</b>	50.2	53	25-59	64.0	68.5	28-81	*
<b>Symptom duration (years)</b>	6.3	4	0.5-38	9.1	8.5	0.5-39	0.018
<b>Women (%)</b>	66			37			0.019
<b>Isolated SFN (%)</b>	69			50			ns
<b>Pain (%)</b>	83			72			ns
<b>Autonomic symptoms (%)</b>	45			76			0.008

Table 3. \*statistical analysis not performed due to different inclusion criteria regarding age. Fisher's exact test was used to compare proportions. Mann-Whitney test was used for comparison of non-parametric values.

#### *5.1.1.1 Metabolic and cardiovascular risk factors*

Eleven percent (5 of 44 patients, data missing for two patients) of the patients included in Study IV smoked, which was significantly less than in Study I, in which 32% (9 of 28 patients, data missing for one patient) smoked ( $p=0.037$ ). There are several differences in the inclusion criterion between Study I and IV such as an upper age limit in Study I and not performing a renewal etiological investigation before inclusion in Study IV, which makes the two study groups non-comparable regarding metabolic and cardiovascular risk factors. However, there was no significant difference in the frequency of included patients with metabolic and cardiovascular risk factors in Study I (28%) compared to Study IV (34%).

In Study I cardiovascular risk factors were defined as follows: pharmaceutical treatment of hypertension or hyperlipidemia, elevated levels of fasting total cholesterol, LDL-cholesterol, or triglycerides, and earlier cardiovascular events. In Study I mean age, gender, pain, and presence of large fiber involvement did not differ significantly between the group with risk factors ( $n=7$ ) and the group without ( $n=18$ ) (four patients were not included in this analysis due to missing data).

In Study IV the definition of metabolic and cardiovascular risk factors was as follows (some patients had more than one risk factor): hypertension, hyperlipidemia, earlier event of ischemic heart- or cerebrovascular disease, non-normal glycemic status and  $BMI > 30$ . In contrast to Study I, when comparing included patients with the above-mentioned risk factors included in Study IV to patients without risk factors in the same study, several differences were identified. The group with risk factors ( $n=16$ ) was older (mean age 68.8 years), had a male predominance (88%), but less pain (50%) compared to the group without risk factors ( $n=30$ , mean age 60.8 years  $p=0.029$ , male 50%  $p=0.023$ , pain 83%  $p=0.036$ ). There were significantly more patients with mixed neuropathy in the group with metabolic and cardiovascular risk factors (81%) compared to the group without risk factors (33%) ( $p=0.0045$ ).

#### **5.1.2 Results of the standardized focused investigation in Study I**

Forty-five patients with seemingly idiopathic SFN underwent our standardized focused investigation (Figure 2). A possible etiology was identified in 12 patients, in which the most frequent cause was IGT (58.3%). Other identified causes are listed in Table 4.

**Table 4. List of diseases identified as a possible etiology by the standardized focused investigation.**

<b>Diseases</b>	<b>Number</b>	<b>Percentage with an identifiable cause (n=12)</b>	<b>Percentage of total investigated patients (n=45)</b>
<b>IGT</b>	7	58.3	15.6
<b>Diabetes mellitus</b>	2	16.6	4.4
<b>Alcohol abuse</b>	1	8.3	2.2
<b>Mitochondrial disease*</b>	1	8.3	2.2
<b>Hereditary neuropathy†</b>	1	8.3	2.2
<b>Total</b>	12	100	26.7

Table 4. \*Genetically verified novel mtDNA mutation. †Clinically suspected HSN.

### **5.1.3 Genetic screening for Fabry disease in patients with idiopathic small fiber neuropathy**

No classical FD mutation was found in the *GLA* gene. However, two possibly pathogenic variants were identified, i.e. D313Y in one female patient in Study I and R118C in one control (Study I) and also in one Swedish patient in Study IV. The control in Study I carrying the R118C was a 58-year-old male anonymous blood donor, from whom we have no further clinical details.

Regarding other genetic alterations of unknown clinical relevance in *GLA*, including the earlier described complex intronic haplotype (141), we found no significant difference among the patients and controls in Study I. In all, 21% (6 of 29) of the patients and 15% of the controls had genetic alterations of unknown clinical relevance in *GLA* in Study I.

Considering that the Sanger sequencing in Study IV was performed in a different laboratory, and there was a variance in which intron regions checked, the results in the two studies are not comparable. The polymorphisms identified in Study IV were not deemed pathogenic by the genetic expert responsible for the analysis.

#### **5.1.3.1 Biochemical analysis and clinical investigations in patients with possible pathogenic variants.**

All six patients with genetic alteration of unknown clinical significance in Study I had normal plasma lyso-Gb<sub>3</sub>, Gb<sub>3</sub> in blood, and no signs of Gb<sub>3</sub> deposits in skin biopsies. Urine-Gb<sub>3</sub> levels

were slightly elevated in a 57-year-old female patient with multiple intronic polymorphisms and in a 49-year-old female patient with D313Y. Both patients had normal  $\alpha$ -GAL activity and MLPA. The patient with the complex intronic polymorphisms had a three years' history of painful isolated SFN with symptoms from extremities as well as trunk and mouth. The patient with the D313Y had a seven years' history of painful symmetric LD-SFN with mild axonal large fiber involvement, with symptoms in both upper and lower extremities. Clinical evaluations with brain MRI (performed in an earlier stage as a part of the clinical etiological investigation), electrocardiography, transthoracic echocardiography, ophthalmologic investigation, and urine protein levels were also normal, indicating absence of clinical signs of FD. Both patients had normal urin-Gb<sub>3</sub> values at a three-year follow-up.

In Study IV the patient with R118C was a 69-year old male with a ten years' history of slowly progressive, mildly painful isolated SFN with symptoms restricted to his feet. He had a normal  $\alpha$ -GAL activity and lyso-Gb<sub>3</sub> in Dried Blood Spot analysis. Clinical evaluation showed no firm evidence of FD, with normal ophthalmologic and skin investigation. The MRI of the heart was normal. He had a mildly impaired kidney function due to earlier urinary traction disease with a post renal obstacle.

#### **5.1.4 Genetic screening of hATTR amyloidosis in patients with idiopathic small fiber neuropathy**

We found no pathogenic mutations in the *TTR* gene.

### **5.2 SURAL NERVE BIOPSY STUDY (STUDY II)**

#### **5.2.1 Demographics and clinical data**

The demographic and clinical data are presented in Table 5. For the normal controls we have no clinical data except for age and sex. There were no significant demographic differences between the groups, except that the duration of symptoms prior to the biopsy was as expected longer for the patients with CIAP compared to the patients with inflammatory neuropathy. There was no significant difference in the proportion of smokers or metabolic and cardiovascular risk factors between CIAP and inflammatory neuropathy patients.

**Table 5. Demographic, clinical and electrophysiological data for patients with CIAP, inflammatory neuropathy and normal controls.**

		<b>CIAP <i>n=10</i></b>	<b>Inflammatory neuropathy <i>n=11</i></b>	<b>Normal controls <i>n=10</i></b>	
<b>Age (years)</b>	mean	54.9	51.4	45.7	ns
	median	57	55	50	ns
	range	25-78	39-59	18-61	
<b>Sex, m:f</b>		5:5	6:5	7:3	ns
<b>Smokers, y:n</b>		4:6	3:7 <sup>b</sup>	<sup>a</sup>	ns
<b>Metabolic/cardiovascular comorbidities, y:n<sup>c</sup></b>		3:7	4:7	<sup>a</sup>	ns
<b>Duration of disease (years)</b>	mean	6.5	3.3	<sup>a</sup>	ns
	median	5.0	1.5	<sup>a</sup>	<i>p&lt;0.05</i>
	range	2-19	0.1-14	<sup>a</sup>	
<b>Need for walking aids, y:n</b>		2:8	3:8	<sup>a</sup>	ns
<b>Neuropathic pain, y:n</b>		5:5	6:5	<sup>a</sup>	ns
<b>Electroneurography index</b>	mean	2.52	5.10 <sup>d</sup>	<sup>a</sup>	ns
	median	2.03	3.35 <sup>d</sup>	<sup>a</sup>	ns
	range	1.26- 6.90	0.38-11.40	<sup>a</sup>	

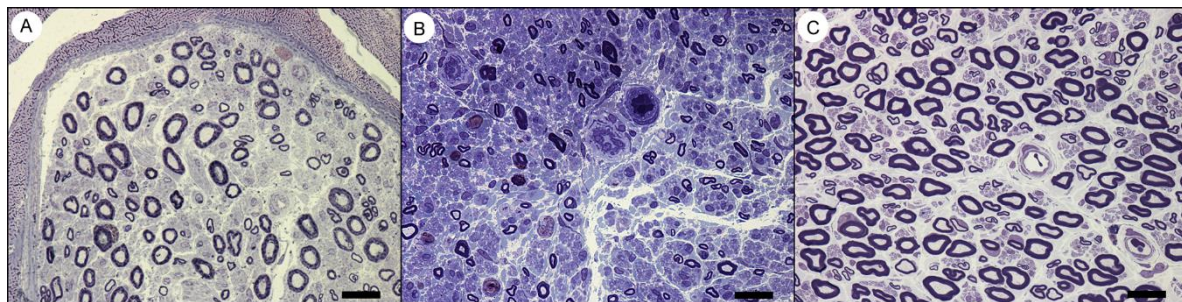
Table 5. <sup>a</sup>Data not available, <sup>b</sup>Data lacking for one patient, <sup>c</sup>Metabolic and cardiovascular comorbidities comprised of hypertension, hypercholesterolemia and moderate levels of white matter changes/ischemia lesions on brain CT/MRI. <sup>d</sup>Data lacking for two patients, n=9.

## 5.2.2 Results of analysis of sural nerve biopsies

### 5.2.2.1 Myelinated and unmyelinated fiber data

There was a significant loss of myelinated fibers both in patients with CIAP ( $p<0.05$ ) and inflammatory neuropathy ( $p<0.001$ ) compared to the normal controls (Table 6). The patients with inflammatory neuropathy tended to have a more severe neuropathy than the CIAP patients, as reflected by the density of myelinated fibers as well as electroneurography results (Table 5), though the differences were not significant. There was no significant difference in the density of unmyelinated fibers between the three groups (Table 6). Subgroup analysis revealed no significant difference in unmyelinated fiber density in CIAP patients with neuropathic pain ( $n=5$ ) or without neuropathic pain ( $n=5$ ).

**Figure 3. Light microscopy picture from a sural nerve fascicle in CIAP (A), inflammatory neuropathy (B) and a normal control (C).**



All scale bars = 20 $\mu$ m. Republished with Permission of PLOS ONE.

### 5.2.2.2 Analysis of the endoneurial microvessels

There was no significant difference in any of the microangiopathic parameters between CIAP patients and normal controls (Table 7). The BLAT and the EA were significantly larger in the group with inflammatory neuropathy than in both the patients with CIAP and normal controls (Table 7). Subgroup analysis showed that the increase of BLAT in inflammatory patients was seen in vasculitic neuropathy and not CIDP (Figure 4). Furthermore, subgroup analysis of patients with CIAP 60 years or older (CIAP<sup>60+</sup>) and patients with CIAP younger than 60 years (CIAP<sup>60-</sup>) showed no significant difference in BLAT, LA or EA compared to normal controls, though a trend towards a larger median BLAT (3.3  $\mu$ m) was found in patients with CIAP<sup>60+</sup> compared to normal controls (median BLAT 2.5  $\mu$ m) ( $p=0.08$ ). However, a clearer influence of age was detected in our entire study population, as there was a significant correlation between BLAT and age ( $p<0.05$ ,  $r=0.15$ ).

**Table 6. Myelinated and unmyelinated density and diameter in sural nerves**

	CIAP <i>n</i> =10	Inflammatory neuropathy <i>n</i> =11	Normal controls <i>n</i> =10
<b>Myelinated fiber density (no/mm<sup>2</sup>)</b>	5,972* (4,186-6,835)	3,113*** (1,506-6,245)	8,648 (7,575-10,337)
<b>Myelinated fiber diameter (μm)</b>	5.6**** (4.3-8.2)	5.7**** (4.5-8.0)	6.7 (4.4-10.5)
<b>Unmyelinated fiber density (no/mm<sup>2</sup>)</b>	26,937 (9,943-42,976)	30,364 (19,990-42,346)	35,143 (23,996-42,145)
<b>Unmyelinated fiber diameter (μm)</b>	1.15 (0.9-1.4)	1.01**** (0.7-1.3)	1.15 (0.9-1.4)

Table 6. Values are presented as median and the 25<sup>th</sup>-75<sup>th</sup> percentiles, \**p*<0.05, \*\*\**p*<0.001, \*\*\*\**p*<0.0001 vs. normal controls

**Figure 4. Basal lamina area thickness in endoneurial microvessels**

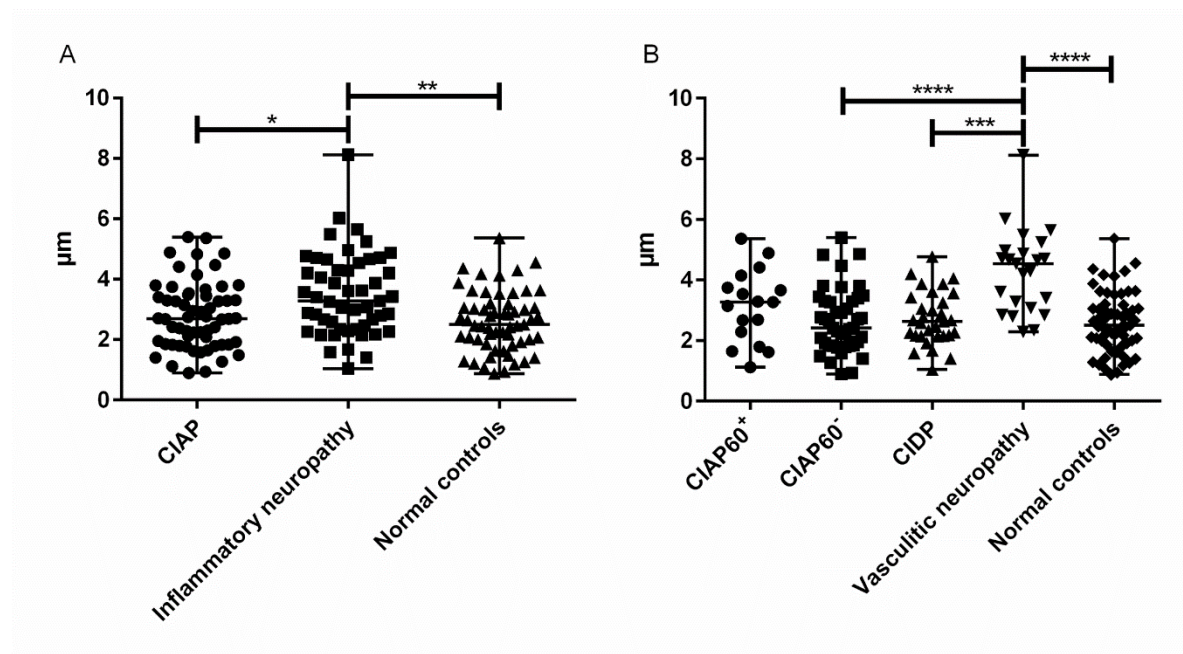


Figure 4. BLAT in sural nerves obtained from patients with CIAP, inflammatory neuropathy and normal controls (A). Subgroup analysis (B). The increase in BLAT in vasculitic neuropathy is not significantly different from the level seen in older patients with CIAP (i.e. those 60 years or older). Vertical bars signify median with range. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001, \*\*\*\**p*<0.0001. Republished with permission from PLOS ONE.

**Table 7. Microangiopathic parameters in sural nerves**

	<b>CIAP <i>n=10</i></b>	<b>Inflammatory neuropathy <i>n=11</i></b>	<b>Normal controls <i>n=10</i></b>
<b>BLAT (<math>\mu\text{m}</math>)</b>	2.7 (1.8-3.5)	3.3** (2.3-4.5)	2.5 (1.9-3.2)
<b>Basal lamina area (<math>\mu\text{m}^2</math>)</b>	81.0 (49.4-116.3)	114.1** (71.0-203.5)	82.8 (54.1-111.2)
<b>Lumen area (<math>\mu\text{m}^2</math>)</b>	7.0 (2.3-16.2)	7.5 (1.6-22.7)	11.8 (3.6-27.1)
<b>Endothelial cell area (<math>\mu\text{m}^2</math>)</b>	28.2 (18.6-35.2)	46.5**** (29.0-78.3)	25.8 (17.4-35.1)
<b>Endothelial cell nuclei (no)</b>	1.5 (1.0-1.9)	2.5 (1.3-3.2)	1.5 (1.2-1.7)
<b>Endothelial cell profile (no)</b>	5.4 (4.8-6.2)	6.8* (5.6-8.2)	5.2 (4.6-5.8)
<b>Periendoneurial cell nuclei (no)</b>	0.7 (0.6-0.9)	1.4**** (1.0-1.8)	0.6 (0.3-0.8)

Values are presented as median and the 25<sup>th</sup>-75<sup>th</sup> percentiles, \* $p<0.05$ , \*\* $p<0.01$ ,  
 \*\*\* $p<0.001$ , \*\*\*\* $p<0.0001$  vs. normal controls.

#### 5.2.2.3 The density and location of autophagy-related structures

The median density of ARS in the sural nerve was significantly higher in patients with CIAP 9,475 no/mm<sup>2</sup> ( $p<0.01$ ) and in patients with inflammatory neuropathy 7,114 no/mm<sup>2</sup> ( $p<0.05$ ) versus normal controls 4,244 no/mm<sup>2</sup>. However, as seen in Figure 5A, there were outliers with high ARS density values in both the group of CIAP patients and the patients with inflammatory neuropathy. Nevertheless, there was still a significant difference of density of ARS in the sural nerves among PNP patients and normal controls even after excluding these outliers (Figure 5B). We found no correlation between the density of ARS in sural nerves and age.

In myelinated axons, the proportion of ARS was significant lower in sural nerve samples taken from CIAP ( $p<0.05$ ) and inflammatory neuropathy ( $p<0.01$ ) patients versus normal



controls (Figure 6). Instead a significant higher proportion of the total number of ARS was found in the Schwann cells in peripheral nerves of CIAP ( $p<0.01$ ) and inflammatory neuropathy ( $p<0.05$ ) patients compared to normal controls. In CIAP patients the ARS were detected especially in the Schwann cells of unmyelinated fibers ( $p=0.001$ ).

**Figure 5. Density of autophagy-related structures in endoneurial microvessels**

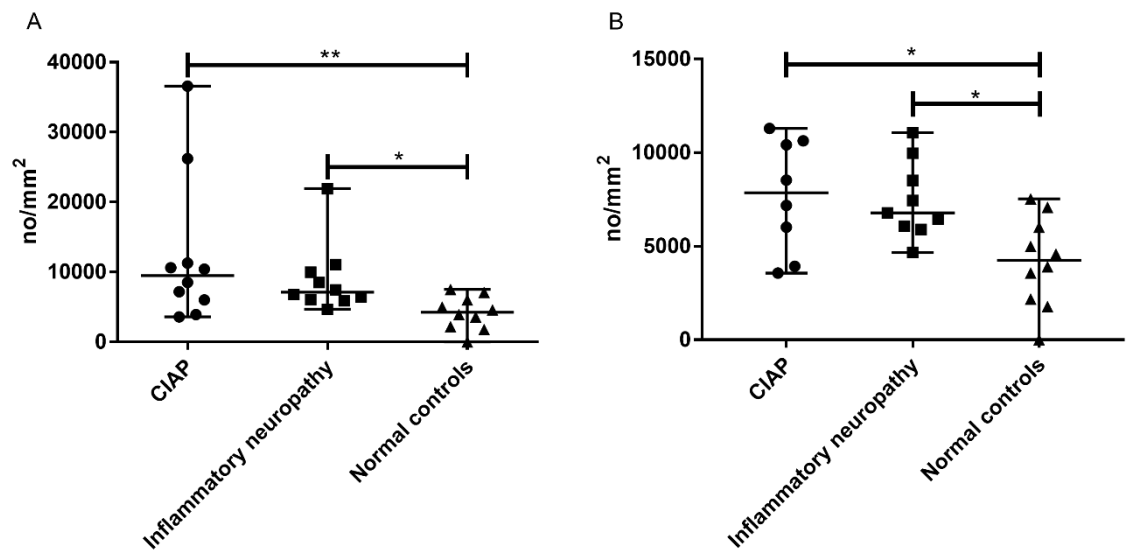


Figure 5. Density of ARS in sural nerves obtained from patients with CIAP, inflammatory neuropathy and normal controls (A), without outliers (B). Note that the scale bar on the Y axis differs between 5A and 5B. The CIAP outliers consisted of a 25-year-old female and a 55-year-old male. The outlier in the inflammatory neuropathy group was a 59-year-old male with a vasculitic neuropathy. Vertical bars signify median with range. \* $p<0.05$ , \*\*  $p<0.01$ . Kruskal-Wallis test, multiple comparison was used for statistical analysis.

**Figure 6. Proportion of ARS found in respective specific location in sural nerves**

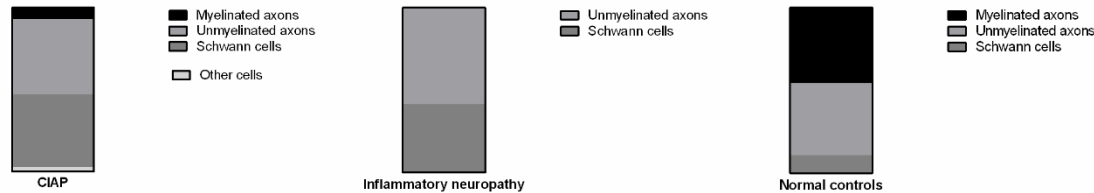


Figure 6. Republished with permission from PLOS ONE.

### **5.3 REGISTER-BASED STUDY (STUDY III)**

We identified 2659 patients with CIAP. Among them 22 patients had MD, 39 patients had AD, and 32 patients had VD, either before or after the diagnosis of CIAP. 13295 age- and sex matched control subjects were also identified. The mean age of patients with CIAP was 65.1 years and there was a male predominance (61%).

#### **5.3.1 Mitochondrial disease, vascular dementia and Alzheimer's disease and the subsequent risk of CIAP (nested case-control study)**

Individuals with MD had a four-fold increased risk of CIAP (OR 4.17, 95% CI 1.27-13.65), while individuals with VD (OR 0.17, 95% CI 0.04-0.69) and AD (OR 0.18, 95% CI 0.06-0.59) had a significantly decreased risk of being subsequently diagnosed with CIAP.

#### **5.3.2 CIAP and the subsequent risk of mitochondrial disease, vascular dementia and Alzheimer's disease (matched cohort study)**

Patients with CIAP and their controls were followed on an average of four years after the index date. There was a nine-fold increased risk of MD among patients with CIAP (Table 8). The risk was more pronounced for younger patients (p-value for interaction 0.05) and for women (p-value for interaction 0.10). Patients with CIAP had a two-fold increased risk of later being diagnosed with VD, but not AD. The association for VD was more prominent, though not reaching significance, during the first year, as well as four years and more after the CIAP diagnosis.

#### **5.3.3 Sensitivity analysis**

In the sensitivity analysis patients with only one hospital visit for CIAP were included and we identified 7551 CIAP patients and selected 37755 controls for them. As in the main analysis, a higher risk of CIAP was still observed in relation to previous MD (OR 3.86, 95% CI 2.05-7.28) and a lower risk of CIAP was observed in relation to previous VD (OR 0.57, 95% CI 0.35-0.93) and AD (OR 0.37, 95% CI 0.24-0.58). However, the subsequent risk for MD (HR 5.37, 95% CI 2.95-9.80) and VD (HR 1.38, 95% CI 1.01-1.87) in CIAP patients was not as prominent as in the main analysis. Complete results from the sensitivity analysis are found in the supplementary information in the manuscript Table S4-S6.

**Table 8. Hazard ratios of mitochondrial disease, vascular dementia and Alzheimer's disease after index date, comparing patients with CIAP to the corresponding controls**

	<b>MD</b>		<b>VD</b>		<b>AD</b>	
	HR	95% CI	HR	95% CI	HR	95% CI
<b>Overall</b>	9.37	4.00-21.93	1.97	1.23-3.16	1.33	0.89-1.98
<b>By sex</b>						
<b>Male</b>	5.38	1.95-14.87	2.03	1.12-3.67	1.68	1.02-2.75
<b>Female</b>	37.24	4.65-298.40	1.89	0.87-4.11	0.92	0.46-1.82
<b>By age at index date</b>						
<b>&lt;=65 years</b>	40.00	5.00-319.79	1.25	0.14-11.18	3.00	0.72-12.55
<b>&gt;65 years</b>	5.13	1.86-14.19	2.03	1.25-3.28	1.25	0.83-1.90
<b>By time after index date</b>						
<b>0-1 year</b>	29.12	3.50-242.05	2.42	0.98-6.00	0.81	0.28-2.34
<b>1-4 years</b>	10.00	2.50-39.98	1.24	0.59-2.63	1.47	0.87-2.48
<b>&gt;4 years</b>	4.20	1.04-16.86	3.41	1.42-8.16	1.46	0.67-3.16

Index date was the first diagnosis of CIAP for cases and the date of selection for controls.

## 6 DISCUSSION

For most neurologists, who work with patients with diseases with a high level of morbidity and even lethal conditions, idiopathic PNP is commonly seen as a benign condition with a relatively small extent of disability. The patients often receive reassuring messages that “they are not in need for specialist care”, “there is nothing we can do for you”, “you will probably not end up in a wheel chair”, “your nerves have gone into an early process of aging” and “there are no effective pharmaceutical treatments anyhow”. However, these statements seldom calm the patient.

Being a PhD student is a research education, but even more important for me it has been a learning about the patients behind the diagnosis PNP. During the last ten years of clinical and research work with patients with idiopathic PNP, it has become more and more clear to me, how important it is to take the time to listen to patients with idiopathic PNP and even more importantly to inform them about their condition. For most of my patients this condition is not as benign as many neurologist colleagues might assume. This realization, apart from my publications, has been one of my most important personal achievements during these years of research.

In the following sections my results will be reflected in relation to previous publications of other research groups.

### 6.1 MORBIDITY OF IDIOPATHIC POLYNEUROPATHY

Patients with idiopathic PNP have a reduced health-related quality of life (3, 6, 151). Idiopathic PNP, especially when pain-associated, has a negative impact on daily activities (7, 259). None of our studies were designed for assessing quality of life or activities of daily living. However, in Study I, a thorough clinical characterization of the included patients with idiopathic SFN was performed and revealed a high morbidity due to pain in this patient group. More than 80% of the patients with idiopathic SFN had continuous neuralgic pain in our study, and about 50% also reported myalgia. The latter being of interest since an increasing number of reports on co-existing SFN and fibromyalgia have been published (98, 260, 261). In an ultrastructural study of nerve fibers in skin biopsies, Doppler et al. reported significant reduced small nerve fiber diameter in patients with fibromyalgia compared to SFN patients and normal controls (261). The authors suggest a different pathogenic mechanism for small fiber dysfunction in patients with fibromyalgia compared to SFN patients (261).

The influence of idiopathic PNP on daily activities such as work has been reported previously in Sweden (7). We found reduced work capacity as a direct consequence of idiopathic SFN in 31% (9 of 29) of our patients in Study I. Schaefer et al. reported a SFN-related negative impact on employment status in about 25% of idiopathic SFN patients in the US.

### 6.2 REEVALUATING PATIENTS WITH IDIOPATHIC POLYNEUROPATHY

If there is to be a value in reevaluating an idiopathic PNP, it clearly will depend on the thoroughness in the original etiological investigation. In retrospective studies where patients

with PNP previously deemed as idiopathic were reevaluated with a structured investigation at a neuropathy center, the proportion of patients judged to have a truly idiopathic neuropathy were expectedly less (156, 174) than the proportion in prospective studies where an initially structured etiological investigation of the patient was performed at base-line with a later reevaluation at follow-up (21, 155, 175).

In Study I a possible etiology was identified in 12 of the 45 (27%) patients with seemingly idiopathic SFN who underwent our standardized focused investigation. The most frequent cause identified was IGT (58.3%, 7 of 12 patients). Interestingly, the glycosylated hemoglobin levels did not differ between the subgroups with and without IGT. Other identified causes in our study were diabetes mellitus in two patients, and single cases each of alcohol abuse, a hereditary cause and MD.

Impaired glucose tolerance and diabetes mellitus are the most frequent diagnoses found in studies reevaluating patients with idiopathic PNP (21, 174, 175). In a two-year follow-up of 28 idiopathic SFN patients, IGT was identified in two patients and diabetes mellitus in four, all of which had normal glucose values at base line (21). Seven IGT cases were identified upon a three-years reevaluation in a Canadian cohort of 228 idiopathic PNP patients (175). Impaired glucose tolerance was also one of the most frequent possible additional SFN-associated conditions identified in the large Dutch study, in which a standardized comprehensive diagnostic algorithm was performed on over 900 patients with pure SFN. Hence the authors suggest the following screening investigations; autoimmune diseases, sodium channel gene mutations, diabetes mellitus including IGT, and vitamin B12 deficiency in all SFN patients already known to have a SFN-associated cause (143).

### **6.2.1 Is impaired glucose tolerance a cause of neuropathy?**

As described in section 1.7.1.1 there is a controversy regarding IGT as an etiology of PNP. Several studies suggest IGT to be a risk factor for neuropathy (20, 86, 87, 161, 184, 185, 194, 195), whereas other controlled studies have not confirmed this hypothesis (152, 196-198).

Another possible theory for the association of IGT with predominant SFN is that foot pain and sensory disturbances in patients with SFN cause physical inactivity and hence weight gain, which then leads to IGT. That could be an explanation as why IGT is overrepresented in the SFN patient group. It would have been interesting to know the BMI curve of the six patients with idiopathic SFN with normal OGTT at baseline, who at the two-years follow-up had developed either IGT or diabetes mellitus in the study by Devigili et al. (21).

Regardless of IGT being a pathogenic cause of idiopathic PNP or not, there is a great value of OGTT to identify IGT in patients with PNP. Lifestyle intervention in patients with IGT and neuropathy does improve IENFD and sural nerve sensory amplitudes (184). Furthermore, an increase in IENFD was described after supervised weekly exercise during one year in patients with diabetes without PNP (199).

Hence, in my opinion OGTT should be included in the etiological investigation of patients with idiopathic SFN or mixed neuropathy, though the identification of IGT should not prevent the remaining etiological investigation to proceed and other potential causes to be considered.

### **6.3 IS THERE A VALUE OF GENETIC SCREENING OF RARE DISEASES IN PATIENTS WITH IDIOPATHIC POLYNEUROPATHY?**

Our results indicate that there is no value of screening for FD or hATTR amyloidosis in patients with idiopathic SFN in a Swedish population unless the patient has disease-specific features or a known family history. However, the divergent reports in earlier genetic screening studies in patients with idiopathic SFN suggest that screening for these disorders might be worthwhile in other populations.

#### **6.3.1 Fabry disease**

The results of previous screening studies for FD in patients with SFN have been conflicting (85, 141, 143), though the largest study including more than 400 patients with isolated SFN in the Netherlands identified no firm cases of FD (143).

In concordance with the Dutch study (143), no classical FD mutations were identified in the *GLA* gene in the included patients in our small pilot study (Study I) or in the larger Nordic cohort in Study IV. We did find patients with a genetic variant of unknown significance (GVUS) in the *GLA* gene in our material. However, in the controlled Study I there was no significant difference in the frequency of GVUS between the patients with idiopathic SFN and the controls (healthy blood donors).

##### **6.3.1.1 D313Y**

The D313Y alteration has been found in 0.45% of subjects in a normal Caucasian population and is described to cause pseudo-deficient enzyme activity (124). However, as mentioned in section 1.5.3.2 there is a dissent whether to consider D313Y pathogenic or not, and whether the patients with D313Y have typical FD phenotype-features or not (125-127, 131-134). D313Y has been suggested to be associated with neurological manifestations such as white matter lesions (128, 129). Further, in a German case series of patients with D313Y ten of 14 patients had symptoms and clinical manifestations of FD, the most common were neurological (stroke and pain) and ocular manifestations (130).

The 49-year-old woman with D313Y identified in Study I had neuropathic pain, though the pain had no specific FD pattern, i.e. pain onset was in adult age and no typical exacerbations provoked by fever, exercise, or heat were described. She had no clinical manifestations suggestive of FD. The only affected biomarker was an elevated urine –Gb<sub>3</sub>, which was normalized at a three-year follow-up.

### 6.3.1.2 R118C

The R118C variant was described as a possible disease-causing late-onset mutation (113) and has been proposed pathogenic in patients with chronic kidney failure (115, 125) and cryptogenic stroke (126, 135, 136). However, a retrospective evaluation of 22 R118C patients showed no shortened life time expectancy or major organ complications (137). The authors suggest the R118C variant to be of low-pathogenicity or non-pathogenic and not to motivate enzyme replacement treatment (137).

In the Swedish cohort in Study IV the R118C variant was detected in a 69-year old male with a ten years' history of slowly progressive, mildly painful isolated SFN. The pain had no typical FD-features. He had normal biomarkers and no firm evidence of FD in clinical evaluation. R118C was also identified in one control, a healthy blood donor, in Study I, for whom we have no further medical information.

### 6.3.1.3 Haplotype IVS0-10C>T [rs2071225] IVS2-81\_-77delCAGCC [rs5903184], IVS4-16A>G [rs2071397], IVS6-22C>T [rs2071228]

There are several reports of a complex intronic haplotype (IVS0-10C>T [rs2071225] IVS2-81\_-77delCAGCC [rs5903184], IVS4-16A>G [rs2071397], IVS6-22C>T [rs2071228]) in *GLA*, also called *GLA* – 10T haplotype, preferentially causing neurological manifestations (140-142), but the *GLA* – 10T haplotype has also been described as the single genetic alteration in *GLA* in a case description of a female with a classical FD phenotype (262).

Tanislav et al. described the complex intronic haplotype in four patients with idiopathic SFN with elevated lyso-Gb<sub>3</sub> and urine-Gb<sub>3</sub> (141). No other firm clinical signs of FD were identified (141). In a screening study of 175 Israeli patients with cryptogenic stroke the complex intronic haplotype was detected in three male and one female patients (142). The male patients had reduced  $\alpha$ -GAL activity and reduced mRNA expression of *GLA*, the latter was also identified in the female patient. Three of the patients had neuropathic pain, i.e. nerve conduction studies were normal (142). In a case series of 15 German patients with the complex intronic haplotype Schelleckes et al. identified neurological manifestations in 13 patients, i.e. five patients with neuropathic pain and WMC or TIA, six patients with neuropathic pain and two patients with stroke (no pain) (140). All patients had normal lyso-Gb<sub>3</sub>, though reduced  $\alpha$ -GAL activity was detected in four of the patients.

The complex intronic haplotype was detected in two of our patients in Study I with completely normal biomarkers. In one patient with another complex intronic haplotype (IVS4-854\_-853delAG, IVS0-12A>G [rs3027585], IVS2-81\_-77delCAGCC [rs5903184], IVS4+68A>G [rs3027589], IVS4-16A>G [rs2071397], IVS6-22C>T [rs2071228]) the urine-Gb<sub>3</sub> level was initially elevated but then normalized. As described in the results no clinical FD features were detected in that patient.

#### 6.3.1.4 *How to interpret the clinical relevance of a genetic variant of unknown significance?*

A definitive diagnosis of FD could be done in a male with a *GLA* mutation and  $\alpha$ -GAL deficiency and at least one of the following criteria; A.  $\geq 1$  of characteristic FD sign/symptom (Fabry neuropathic pain, cornea verticillata or clustered angiokeratoma), B. an increase of lyso-Gb<sub>3</sub> and C. a family member with a FD diagnosis carrying the same mutation (263). For a female patient the diagnostic criteria are the same, except for that  $\alpha$ -GAL activity could be normal (263). All patients with a non-specific FD feature, such as cardiac left ventricular hypertrophy, stroke at young age or proteinuria, not fulfilling the above-mentioned criteria for a definitive FD diagnosis, have a *GLA* GVUS. To confirm their FD diagnosis ultrastructural analysis of a biopsy from an affected organ (i.e. heart or kidney) with findings of typical FD storages should be performed (263, 264). Van der Tol et al. have proposed a diagnostic algorithm to approach the individuals with a GVUS in the *GLA* gene (264).

Furthermore, Biegstraaten et al. suggest that SFN should be considered to be due to FD only in patients with SFN showing typical FD features; i.e. a history of neuropathic pain in the hands and/or feet, with the onset of pain in either childhood or adolescence, or a course that is characterized by ongoing pain with exacerbations that are provoked by fever, exercise, or heat (265).

We did not consider any of our identified patients with a GVUS to have a FD due to lack of consistent pathological biomarkers, no other firm clinical FD manifestations, no family history of FD, and most importantly lack of typical FD pattern in their pain description. However, considering the variance in the description of patients clinical FD manifestations in other case series of patients with D313Y, R118C and maybe also the complex intronic haplotype, these patients should go through a careful clinical evaluation and a case-to-case judgment as to the clinical relevance of the *GLA* GVUS.

#### 6.3.2 Hereditary ATTR amyloidosis

Recently two screening studies of hATTR amyloidosis in patients with idiopathic PNP have been published, one from the US and one from China (84, 85). Levine et al. screened 47 US patients with idiopathic SFN or mixed neuropathy and reported no amyloidogenic mutations in the *TTR* gene (84). In the Chinese study three cases of hATTR amyloidosis (all Ala97Ser) were identified in a cohort of 100 idiopathic pure SFN patients (85).

We found no pathogenic mutation in the *TTR* gene in the Nordic cohort in Study IV among 155 screened patients with idiopathic pure SFN or mixed neuropathy.

### 6.4 IS MICROANGIOPATHY INVOLVED IN THE PATHOGENESIS OF IDIOPATHIC POLYNEUROPATHY

We found no significant difference in any microangiopathic parameter in the endoneurial microvessels in the sural nerves of CIAP patients compared to normal controls. Hence, we could not confirm the reports from Teunissen et al. and Hube et al. suggestive of



microangiopathy being present in sural nerves in CIAP patients (187, 219). However, Teunissen et al. found no significant difference when comparing BLAT in CIAP patients to that in autopsy controls using univariate analysis ( $p=0.08$ ). The study by Hube et al. lacked negative controls and furthermore it was not an ultrastructural study, as the analysis was performed by light microscopy. Hence the positive results from these studies were based on the observation that increased BLAT seen in CIAP was in the same range as in patients with diabetic neuropathy i.e. the positive controls (187, 219).

Moreover, Teunissen et al. investigated the occurrence of peripheral arterial disease in their group of CIAP patients (219). Seven of the 18 CIAP patients in their study had abnormal ankle brachial index and sural nerve BLAT was significantly enlarged in those patients. However, the authors did not report the subgroup analysis comparing BLAT in CIAP patients with normal ankle brachial index, with BLAT of HMSN 2 patients and autopsy cases (219). Increased BLAT has been described in patients with peripheral arterial disease (218), hence one cannot rule out that the peripheral arterial disease in the CIAP patients partly contributed to the increased BLAT reported by Teunissen et al. (219).

Another possible explanation for the divergent result between us and the above-mentioned studies is that our CIAP patient group was younger (mean age 54.9 years) compared to the other two studies (mean age 63.0 years (219) and 61.1. years (187)). Our subgroup analysis showed a trend towards a larger median BLAT (3.3  $\mu\text{m}$ ) in CIAP patients older than 60 years compared to normal controls (median BLAT 2.5  $\mu\text{m}$ ) ( $p=0.08$ ). Furthermore, we identified a positive correlation between age and BLAT in the whole cohort of patients and controls ( $p<0.05$ ,  $r=0.15$ ). Though, this is inconsistent with several previous studies reporting no correlation between BLAT and age (208, 215, 219, 266). However, Jacobs and Love reported prominent reduplication of the basement membranes of the vasa nervorum from the sixth decade and onwards (267).

Abnormalities in microvessels in sural nerve biopsies obtained from patients with diabetic neuropathy are well documented (204-215). However, similar changes have also been detected in patients with HMSN 1. Bradley et al. reported increased EA and BA in HMSN 1 patients compared to controls (204). These endothelial findings in HMSN 1 patients suggest microangiopathy to be secondary to the neuropathy, rather than causative for it (204). Arguments against this hypothesis are that basal membrane thickness precedes diabetic neuropathy (208, 210, 212), and that repeated axonal de- and regeneration in rat nerves does not increase endoneurial basal membrane reduplication in microvessels (268).

In view of the mounting evidence of an association between the metabolic syndrome and CIAP (20, 86, 87, 152, 158, 161, 182-186), the hypothesis of microangiopathy in vasa nervorum as a pathogenic cause of CIAP is appealing. However, considering the inconsistent results in the small and methodologically limited studies exploring this hypothesis, there is no firm evidence that microangiopathy contributes to the pathogenesis or even exists in endoneurial microvessels in sural nerves in CIAP patients. Furthermore, considering the results by Teunissen et al., if microangiopathy does exist, it may in part be caused by

ischemia due to co-existing peripheral arterial disease (219). The limitations in Study II are discussed in section 6.7. Further studies addressing this hypothesis are required and a possible way to proceed will be discussed in the chapter of Future Perspectives.

#### **6.4.1 The unexpected findings in vasculitic neuropathy**

The patient group that unexpectedly did show signs of endoneurial microangiopathy was the patients with vasculitic neuropathy, in whom BLAT and EA were significantly increased compared to normal controls. The elevated BLAT remained significant after exclusion of vessels with prominent periendothelial cytoplasmic structures, so the increased BLAT was not merely due to cellular infiltration in the vessels. Another possible explanation for enlarged BLAT in vasculitic neuropathy could be edema in the microvessels, but apparent structural gaps in the endothelial junctions to support this hypothesis were not observed. Similar structural abnormalities with thickened and reduplicated basal lamina in the endoneurial microvessels have been described in two old case reports of one patient with Granulomatosis with polyangiitis and one patient with polyarteritis nodosa (269, 270). However, changes in vessel structure in patients with vasculitic neuropathy have mainly been studied in epineurial (271, 272), rather than endoneurial vessels which were the blood vessels studied by us.

#### **6.4.2 The possible association between microangiopathy in the PNS and CNS**

Holding on to the hypothesis that microangiopathy does exist in the patients with CIAP, the following question arises, i.e. whether there is an association between microangiopathy in the PNS and CNS, clinically manifested as CIAP and VD respectively?

As mentioned above, metabolic risk factors are associated with CIAP (20, 86, 87, 152, 158, 161, 182-186). In addition, metabolic risk factors such as diabetes and hypertension are associated with an increased risk of WMC (222, 223), which is a risk factor for VD (220, 221). The report that patients with diabetes mellitus type 2 and PNP had significant more WMC compared to non-neuropathic diabetes patients, even after adjusting for age and duration of diabetes, support the idea of a common microangiopathic pathogenesis in the PNS and CNS (227). Furthermore, there was no significant difference between the patients with or without PNP with regards to the presence of carotid atherosclerosis, which favours a micro- rather than a macroangiopathic process (227).

In our nation-wide register-based study (Study III) we explored if there was an association between CIAP and VD. We found that patients with CIAP had a two-fold increased risk of later being diagnosed with VD. The association for VD was more prominent during the first year, as well as four years and more after the CIAP diagnosis. Surveillance bias, i.e. in this case, an increased probability of noticing and further examining cognitive impairment during the investigation of walking difficulties in patients with CIAP, might partially explain the association seen in the first year after CIAP diagnosis. However, the circumstance that the

association gets stronger again after four years might indeed indicate that microangiopathy in the PNS and CNS do co-exist.

The decreased risk of being subsequently diagnosed with CIAP seen in both patients with VD and AD was probably due to negative detection bias i.e. walking difficulties in patients with dementia tend not to get further investigated.

## **6.5 THE POTENTIAL ROLE OF AUTOPHAGY IN POLYNEUROPATHY**

We found an increased density of ARS in sural nerves in patients with CIAP ( $p<0.01$ ) and inflammatory neuropathy ( $p<0.05$ ) compared to normal controls.

Our collaborators in Linköping have earlier shown an increased density of ARS in the posterior interosseous nerve in patients with diabetes mellitus type 1 compared to patient with diabetes mellitus type 2 and controls, though in the latter not reaching significance ( $p=0.06$ ) (244). In agreement with the report of Osman et al. (244), our results indicate ultrastructural signs of the autophagy pathway being present in human peripheral nerve.

The activated autophagy pathway seems not to be a normal age-related process, since we did not find a correlation between the density of ARS and age. Nor did we find a correlation between the duration of CIAP and the density of ARS, suggesting that the activation of the autophagy pathway appears to be an early ongoing process in CIAP and not merely an end-stage one.

Regarding the localization of ARS in the sural nerve, significantly more ARS were encountered in Schwann cells in CIAP and inflammatory neuropathies compared to normal controls. Interestingly, in patients with CIAP the ARS were preferentially located within Schwann cells of unmyelinated axons. In both CIAP and inflammatory neuropathy patients, the proportion of ARS was significantly less in myelinated axons versus normal controls. The same pattern of localization was described by Mohseni et al. in a follow-up study in sural nerve biopsy in patients with diabetes mellitus type 2 (216). The proportion of ARS encountered in myelinated axons was significantly reduced (almost reaching zero) in the second sural nerve biopsy obtained after eleven years (contralateral side) (216). The proportion of ARS in Schwann cells had increased though not reaching significance. In parallel, the myelinated fiber density had declined significantly (216).

Obviously the increase in the density of ARS was not specific to CIAP patients considering that a similar, though a somewhat milder pattern of autophagy alterations was also seen in patients with inflammatory neuropathy. The cause of the different level and location of ARS in sural nerves of patients with PNP compared to normal controls is not known. Obviously, we cannot exclude the possibility that the lower number of ARS in the myelinated axons in CIAP patients, merely is due to axonal degeneration. However, we found no correlation between myelinated fiber density and the density of ARS in CIAP patients.

The question whether autophagy is a pathogenic factor in idiopathic neuropathy or whether the findings are merely a consequence of the neuropathic process, remains to be solved. Further immunohistochemistry and protein analysis studies are required to better understand the process of autophagy and its potential role in CIAP.

## **6.6 IS THERE AN ASSOCIATION BETWEEN MITOCHONDRIAL DISEASE AND IDIOPATHIC POLYNEUROPATHY?**

In Study III we showed that individuals with MD had a four-fold increased subsequent risk of CIAP and among patients with CIAP, there was a nine-fold increased subsequent risk of MD. Our finding of a firm association between MD and CIAP was anticipated, as previous studies reported a PNP prevalence of 12-45% among patients with MD (245-247). However, prior evaluation of an association between CIAP and MD on a population level has to our knowledge not been performed.

Polyneuropathy co-existing with MD might not be categorized as idiopathic, but rather as a mitochondrial syndrome-related peripheral nerve affection. Though, as mentioned in section 1.7.3 mitochondrial dynamics are crucial for the energy requirements in peripheral axons (250). Therefore, the hypothesis of idiopathic PNP being caused by mitochondrial dysfunction in peripheral nerve axons, either as a consequence of energy depletion or as a disturbed distribution of mitochondria, requires further studies.

## **6.7 LIMITATIONS**

Two general limitations of this thesis (except for Study III) are the relatively small patient material and the predominant retrospective set-up.

### **6.7.1 Limitations in Study I and IV**

#### *6.7.1.1 The limited patient material, retrospective set-up and inclusion process*

Study I was a pilot study with a small patient material. However, with the expanded number of included patients in Study IV through the Nordic collaboration, the genetic screening results regarding FD from Study I was confirmed. For both Study I and IV the inclusions were retro- as well as prospective. The possibility of identifying patients through registers and reviewing of medical records enlarged the patient group that potentially could be included. In Study I a widened and renewed clinical and laboratory reevaluation of each retrospectively identified patient was performed confirming the SFN to be truly idiopathic. The same thorough assessment was not done in Study IV, which might be seen as a limitation, on the other hand that might be more similar to an actual situation clinicians face in their day-to-day practice. The approach of including patients with an idiopathic SFN, may have resulted in exclusion of patients with a known possible SFN-associated disease as well as a hATTR amyloidosis or FD, such as a patient with FD and IGT. However, since the large genetic screening study for FD in the Netherlands did include patients with other disease-associated etiologies to SFN (143), wider inclusion criteria probably would not have affected our results, at least for the FD screening.

### *6.7.1.2 Diagnosis of small fiber neuropathy*

As earlier discussed there are many methods to assess small fiber function. The choice to use QST as the method for diagnosing SFN in our studies (Study I and IV), was mainly based on the accessibility and that QST is the available method in clinical practice in Sweden. There are no accredited laboratories in Sweden performing IENFD analysis in skin biopsies in clinical daily routine work. Quantitative sensory testing has been found to be strongly correlated with IENFD in detecting SFN according to the EFNS guidelines (48, 49). Skin biopsy may miss patients with dysfunction rather than reduced number of nerve fibers (51). However, one limitation with QST is the inability to differ between PNS and CNS pathology. To compensate for the shortcoming of QST sensitivity, we required that all included patients with isolated SFN, also had a clinical evaluation aimed at minimizing the risk of a CNS disorder being a cause of their sensory symptoms.

### *6.7.1.3 The implication of fiber heterogeneity*

Another possible limitation of Study I and IV is the heterogeneity of nerve fiber affection in the included patients, allowing both isolated SFN and mixed neuropathy i.e. combined SFN and axonal large fiber neuropathy. The rational for including mixed neuropathy in Study IV is easier to assent, considering that the genetic screening included also hATTR amyloidosis in which large fiber neuropathy is a common feature. Large fiber affection in FD does occur, but is uncommon. We therefore cannot exclude the possibility of an alternative result in FD screening, if only patients with pure SFN were to be included. On the other hand our results were consistent with the large Dutch study screening for FD in a cohort of pure SFN (143).

### *6.7.1.4 The controls in Study I*

In Study I we lack controls for the standardized focused investigation. The controls for the genetic study were age- and sex matched. However, we did not have any other clinical information about possible co-morbidities and cardiovascular risk factors of the controls.

## **6.7.2 Limitations in Study II**

### *6.7.2.1 Limited patient material and selection bias*

Considering the invasive nature of a nerve biopsy with the risk of persistent sensory disturbance and neuropathic pain and it not being a regular part of the etiological investigation of idiopathic PNP nowadays, we considered it of great value performing this study despite the limited patient- and control material and its retrospective set-up. Naturally, a limitation with the retrospective set-up is the selection bias i.e. patients getting their final diagnosis only after the neuropathological evaluation of the sural nerve biopsy.

### *6.7.2.2 The young CIAP patient group*

CIAP is considered to rarely start before the 6<sup>th</sup> decade of life. Our CIAP patient group was relatively young, though only two patients were younger than 50 years. Considering the difficulty in differentiating sporadic axonal HMSN 2 from CIAP patients, one cannot

completely rule out the possibility of a patient with a sporadic hereditary PNP being included in our CIAP group.

#### *6.7.2.3 Limitation in methodology*

The morphological methods used to measure the parameters of fibres, microangiopathy and autophagy structures in sural nerves in the present study included a certain extent of subjectivity in the assessment. However, this does not differ from previous morphological studies. A limitation of the assessment of microangiopathy is the lack of a golden standard how to perform the evaluation.

#### *6.7.2.4 The normal controls*

The normal controls consisted of patients with symptoms of distal weakness and/or sensory symptoms from the lower limbs, henceforth they were not truly healthy controls, some of them ended up with the final diagnosis of a neurological disease being excluded, others with a CNS disorder or a strictly motor PNP. The reason why they were included into the study as normal controls was that none had sensory PNP, hence their sural nerve was considered as normal.

A further limitation was the lack of information about smoking habits and possible metabolic or cardiovascular risk factors and associated comorbidities for the normal controls.

### **6.7.3 Limitations in Study III**

The register-based nature of the study imposes several limitations.

#### *6.7.3.1 Diagnostic errors*

The CIAP diagnosis was based on the ICD-10 codes for unspecified PNP (G62.9) or idiopathic progressive PNP (G60.3) in combination with the exclusion of “all” possible disease-associated etiologies of PNP. However, without verifying all the medical records for CIAP, there indeed is a risk of misclassification. We cannot rule out the possibility of some remaining inflammatory or hereditary polyneuropathies in the CIAP group, hence using the definition of at least 2 separate visits with the same ICD-code at least reduces that risk.

There might also be some degree of misdiagnoses between AD and VD. However, we chose not to include the ICD-10 code for unspecified dementia (F03), and in the validation analysis we found that AD drugs were prescribed for 66% of the AD patients whereas only 18% of the VD patients.

Furthermore, some of the ICD codes used for MD are not entirely specific. However, such misclassification should be non-differential between patients with CIAP and the controls.

#### *6.7.3.2 Selection bias*

As mentioned above, to further improve the diagnostic accuracy of CIAP we restricted the analyses to patients with at least two visits for CIAP. This design however introduces a

selection bias since the Swedish patient register does not include primary care visits. Many patients with CIAP, might only visit a hospital-based outpatient specialist care, i.e. a neurologist, once, hence our definition might lead to a selection of patients in requirement of more specialist care. Therefore, we conducted a sensitivity analysis where we included all patients with at least one hospital visit for CIAP, obtaining largely similar results as in the main analysis.

#### *6.7.3.3 The lack of detailed phenotypic and genetic information*

The lack of validation of diagnosis, phenotypic and if possible genotypic information is a limitation especially in MD patients, but also in VD. A future study should preferentially include validation of the diagnosis by reviewing medical records.

## 7 CONCLUSIONS

An alternative title to this thesis would be *Exploring idiopathic polyneuropathy – still idiopathic*. However, I feel that I have managed to shed some light as to the potential causes of idiopathic polyneuropathy.

Though based on a small patient material, in patients with seemingly idiopathic predominant SFN, a standardized focused investigation is of importance to reveal possible etiologies of SFN. Impaired glucose tolerance was the most common identifiable cause in our study.

Screening for hereditary ATTR amyloidosis and Fabry disease in patients with idiopathic SFN or mixed neuropathy without any additional disease-specific symptoms or clinical characteristics in a Swedish population seems not to be of value in a clinical setting.

We could not confirm the earlier described findings of microangiopathy in endoneurial microvessels in sural nerves in patients with CIAP.

The density of autophagy-related structures in the sural nerve was significantly increased in patients with CIAP and inflammatory neuropathy compared to normal controls. Whether the altered autophagy pathway is a consequence or a cause of the neuropathy in patients with idiopathic polyneuropathy is not clear.

In a large register-based population study a strong association between mitochondrial disease and idiopathic polyneuropathy was identified.

We found a reduced risk of CIAP among individuals with vascular dementia or Alzheimer's disease, which might be due to a reduced surveillance of symptoms of PNP among dementia patients.

Patients with CIAP had an increased subsequent risk of vascular dementia. This finding might support the hypothesis that microangiopathy in the PNS and the CNS do tend to co-exist.



## 8 FUTURE PERSPECTIVES

As a clinician I will start this section with some mainly clinical reflections regarding the group of idiopathic painful SFN patients, with PNP onset often at a relatively young age.

We have no SFN patients with verified sodium channel mutations in our outpatient clinic. This is surely not due to Na<sub>v</sub>1.7-9 mutations being absent in the Stockholm population, rather an indication of too few patients being referred for genetic screening for Na<sub>v</sub> mutations at our department. Does it matter if we identify the SFN patients with sodium channel mutations or not? Besides being able to provide the patients an explanation for their condition, some of these patients might be offered an effective pain reduction with lacosamide based on the results of the newly completed but not yet published phase 3 study of lacosamide in patients with gain-of function Na<sub>v</sub>1.7 mutations ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). A further interesting study is the ongoing randomized double-blinded placebo-controlled clinical trial with treatment with IVIG for idiopathic SFN, with pain relief as the primary outcome (173). Hence, an upcoming challenge in the clinical work is to identify which patients to screen for sodium channel mutations and which patients might have an immunologically mediated SFN, and therefore could be candidates for immunological treatment.

As mentioned in the discussion, the hypothesis of microangiopathy contributing to the pathogenesis of CIAP is very appealing. However, several challenges will be encountered in future studies raising this question. The ideal study design with a prospective sural nerve biopsy study in CIAP patient would be difficult to perform since sural nerve biopsy is not a part of the regular etiological clinical investigation in CIAP patients. Also including a healthy control group would be impossible at least in Sweden today due to current ethical regulations. Henceforth, the retrospective set up and hence selection bias is difficult to avoid. The task is rather to minimize the subjectivity in measurements reporting microangiopathy and rather to identify a more specific objective histopathological marker representative for it. Teunissen et al. reported that the increased basement membrane area in CIAP patients consisted mainly of collagen (219), hence a immunohistochemistry study of collagen may be a possible way to resolve the uncertainty whether CIAP is associated with microangiopathy in the endoneurial microvessels.

One way to further explore the possible co-existence of microangiopathy in the PNS and CNS is to investigate whether CIAP patients have more WMC, visualized by a brain MRI, compared to controls without CIAP. Though such an association would not constitute proof of a common pathogenesis of axonal degeneration in the PNS with WMC in the CNS, it may indicate a shared disease mechanism. Our research group has an on-going prospective MRI study in patients with CIAP.

Study II has in accordance with previous work by our collaborators in Linköping provided evidence of the autophagy machinery being active in the human peripheral nerve, though our study was not designed to show if an active autophagy machinery in the PNS is the cause, or rather the consequence of axonal degeneration in CIAP. Furthermore, the morphological

pattern of localization and level of the density of the ARS differ between patients with PNP versus normal controls. However, so far EM has been the only method to detect ARS in human peripheral nerves. Further immunohistochemistry and protein analysis studies to explore the autophagic flux, i.e. the turnover of degraded proteins, are warranted to better understand the process of autophagy and its potential role in PNP in general and particularly in idiopathic PNP. For this un-fixated fresh or frozen nerve tissue is needed, which has not been available in the previous studies.

An unexplored field is the potential role of mitochondrial pathology and disturbed mitochondrial dynamics in the axons in idiopathic PNP. The pathology of mitochondrial function in some defined hereditary PNP is described in the background section. Furthermore, in an animal model of toxic neuropathy loss of total mass and changed morphology of mitochondria in axons have been shown (256). Mitochondrial dysfunction has been suggested to be involved in the pathogenesis of diabetic neuropathy (253-255). Therefore, an ultrastructural study of potential pathological mitochondria morphology in idiopathic PNP in human peripheral nerves would be of great interest and consequently also immunohistochemistry and protein analysis studies.

## 9 POPULÄRVETENSKAPLIG SAMMANFATTNING

Polyneuropati (PNP) innebär funktionsstörning i nervtrådarna i det perifera nervsystemet (PNS) dvs. de nerver utanför hjärnan och ryggmärgen som styr muskler, känselsinne och de icke-viljestyrda aktiviteterna i kroppens olika organ. PNP kan kategoriseras utifrån vilka nervtrådar (axon) som är drabbade, de grova myeliniserade nervtrådarna (grovtrådsneuropati) eller de tunna nervtrådarna (fintrådsneuropati). Grovtrådsneuropati kan vidare delas in i axonal eller demyeliniserande typ, beroende på om det är axonet (nervtråden) eller myelinet (fettskidan) i de perifera nerverna som främst är drabbade.

Beroende på vilka nervtrådar som är påverkade får patienterna varierande grad av symtom såsom känselnedsättning, domningar, smärta och muskelsvaghet i främst fötter, underben och händer samt balans- och gångsvårigheter. PNP är en vanlig åkomma och förekomsten i hela befolkningen är ca 1,6 %. Tillståndet är vanligare i högre åldersgrupper och över 60 års ålder är förekomsten runt 6,5 %. Studier har påvisat att en ökad andel av patienter med PNP har sänkt livskvalitet och att sjukdomen kan ha negativ påverkan på dagliga aktiviteter i hemmet, på fritiden och på arbetet. Patienter med PNP har en ökad risk för fall och fall-relaterade skador.

Hos ca 25 % av patienter med PNP lyckas man inte påvisa någon bakomliggande orsak trots en omfattande utredning, varvid diagnosen idiopatisk PNP ställs. Detta är vanligast vid fintrådsneuropati samt vid en så kallad kronisk idiopatisk axonal polyneuropati (CIAP), en symmetrisk axonal PNP som främst drabbar känselnerver med långsam försämringstakt och som förekommer framför allt hos äldre patienter. Avsaknad av en känd bakomliggande orsak betyder att patienter med idiopatisk PNP förblir obehandlade, varför en ökad ansträngning för att identifiera bidragande faktorer är högst önskvärd. Det finns olika hypoteser till patogenesen, dvs. den sjukdomsalstrande orsaken, av idiopatisk PNP, såsom underdiagnostisering av ärftliga sjukdomar där PNP ingår som delsymtom, metabola riskfaktorer (högt blodtryck, förhöjda blodfetter, fetma) med bidragande småkärslsjukdom, nedbrytande orsaker i nervtrådarna (axondegeneration) samt funktionsstörning i mitokondrierna (cellens energifabrik).

Den här avhandlingen bygger på fyra delstudier där vi med olika metoder har belyst möjliga patogenetiska faktorer till idiopatisk PNP.

Fabrys sjukdom är en ärftlig sjukdom där man till följd av en förändring i arvsmassan har brist på ett protein som bryter ned särskilda fetter i cellerna. När detta inte fungerar får man inlagringar i sårbara organ såsom hjärta, njurar, ögon och perifera nerver. I Studie I undersöktes om sent debuterande Fabrys sjukdom kan vara en bakomliggande orsak till idiopatisk fintrådsneuropati hos unga till medelålders patienter. Vi avsåg även att genom en standardiserad strukturerad utredning identifiera möjliga bakomliggande orsaker till idiopatisk fintrådsneuropati. En möjlig bakomliggande orsak till PNP identifierades hos 12 av de 45 patienter (27 %) som genomgick en omfattande klinisk standardiserad utredning. Den vanligaste orsaken var nedsatt glukostolerans som hittades i 58 % (7 av 12 patienter). De

resterande 33 patienterna med idiopatisk PNP erbjöds att genomgå genetisk screening för Fabrys sjukdom, varav fyra patienter avböjde. Genetiska variationer av oklar klinisk betydelse identifierades hos 6 av 29 patienter med genuin idiopatisk fintrådsneuropati. Frekvensen var densamma hos friska kontroller. Ingen av patienterna med genetiska varianter hade avvikelser i blod eller urinprov som talade för Fabrys sjukdom och inte heller någon påverkan på andra organ som är typiska vid Fabrys sjukdom. Sammanfattningsvis så talar denna pilotstudie för att idiopatisk fintrådsneuropati hos unga till medelålders patienter i Sverige inte orsakas av Fabrys sjukdom samt att en vanlig möjlig bidragande orsak till fintrådsneuropati är nedsatt glukostolerans.

Flera studier har visat ett samband mellan metabola riskfaktorer och CIAP. Hos patienter med diabetes mellitus och PNP har man visat att det finns en ökad mängd förändringar i små mikrobldkärll som försörjer nerver, så kallad mikroangiopati. I Studie II ville vi undersöka om det fanns liknande förändringar hos patienter med CIAP. Sparat material från nervbiopsier från tio patienter med CIAP jämfördes med elva ålders- och könsmatchade kontroller med inflammatorisk orsak till PNP och tio kontroller utan känd PNP. Genom fotografering av preparaten i ljus- och elektronmikroskop gjordes analyser på digitala bilder med hjälp av mjukvaruprogram. Det fanns ingen signifikant skillnad i de undersökta mikroangiopatiska parametrarna mellan patienter med CIAP och normala kontroller. Vidare undersökte vi om det fanns en ökad förekomst av autofagi-relaterade strukturer hos patienter med CIAP jämfört med kontroller. Autofagi är cellens normala städmaskineri där cellen kan göra sig av med skadade eller icke-fungerande celldelar. Vi fann en ökad förekomst av autofagi-relaterade strukturer hos både patienter med CIAP och de inflammatoriska patienterna jämför med de normala kontrollerna. Sammanfattningsvis stödjer våra resultat inte hypotesen att CIAP primärt är orsakat av mikroangiopati i små mikrokärll i perifer nerv. Vi har kunnat visa att autofagi-processen är aktiv i perifera nerver hos människor, men det är inte klarlagt om den ökade mängden autofagi-relaterade strukturer vid CIAP är till följd av PNP eller om det är en del av orsaken till PNP.

Utifrån sambandet mellan metabola riskfaktorer och CIAP i PNS samt ett liknande samband mellan metabola riskfaktorer och vaskulär demens i det centrala nervsystemet (CNS), dvs. hjärnan, så undersökte vi i en populationsbaserad registerstudie (Studie III) kopplingen mellan CIAP och vaskulär demens, Alzheimers sjukdom (inkluderad som en negativ kontroll, inget förväntat samband) samt mitokondriell sjukdom. Polyneuropati är vanligt förekommande hos patienter med mitokondriella syndrom, men sambandet har inte tidigare undersökts i populationsstudier. Patienter med CIAP identifierades genom utvalda diagnoskoder för PNP (ICD-10) i Svenska Patientregistret under perioden 2001–2010. För att studera patienter med idiopatisk PNP exkluderades från studiebasen individer med ICD koder för andra sjukdomar som är kända för att bidra till patogenesen till PNP såsom t.ex. diabetes mellitus. I en fall-kontroll studie inkluderades 2659 patienter med CIAP och 13295 ålders- och könsmatchade kontroller. Därutöver utförde vi en kohortstudie där patienterna med CIAP följdes i genomsnitt i fyra år. Sammanfattningsvis så fann vi en mycket stark koppling mellan mitokondriell sjukdom och CIAP. Individer med mitokondriell sjukdom hade en fyrfaldig

ökad risk för CIAP och patienter med CIAP hade en niofaldig ökad risk för mitokondriell sjukdom. Individer med vaskulär demens och Alzheimers sjukdom hade en uttalat minskad risk för CIAP. Den minskade risken för PNP-diagnos bland individer med demens skulle kunna bero på en negativ detektionsbias, dvs. att denna patientgrupp utreds mindre för sina gång- och balanssvårigheter. Patienter med CIAP hade en tvåfaldig ökad risk för vaskulär demens jämfört med individer utan CIAP vilket ger ett visst stöd för den bakomliggande hypotesen att samexistens av mikroangiopati i PNS och CNS manifesteras kliniskt som CIAP och vaskulär demens.

Hereditär ATTR amyloidosis (även kallad Skellefteåsjukan eller TTR-FAP) är en ärftlig sjukdom där en förändring i arvsmassan för proteinet transtyrelin (TTR) gör att kroppen inte kan bryta ner TTR utan det lagras i olika organ såsom hjärta, njurar, ögon och perifera nerver. I Sverige är sjukdomen vanligast i Västerbotten och Norrbotten och debuterar ofta i äldre medelåldern, vanligen efter 50 års ålder. I Studie IV undersöktes värdet av genetisk screening för Fabrys sjukdom och hereditär ATTR amyloidosis i en klinisk utredning av patienter med idiopatisk fintrådsneuropati i Norden med nio deltagande neurologiska center från Danmark, Finland, Norge och Sverige. 172 patienter med idiopatisk fintrådsneuropati med och utan grovtrådspåverkan inkluderades mellan oktober 2015 och februari 2017. Sjutton patienter exkluderas pga. olika identifierade orsaker till PNP som framkom senare under inklusionsprocessen. Genetisk screening för Fabrys sjukdom och hereditär ATTR amyloidosis utfördes hos 155 patienter. Inga säkra fall av respektive sjukdom identifierades. Genetisk screening av patienter med idiopatisk PNP utan något ytterligare sjukdomsspecifikt symtom eller klinisk karakteristik i en nordisk befolkning bedöms inte vara av värde i den kliniska vardagen.

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